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Activity in Single Hippocampal Pyramids

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Abstract

EULER, C. v. and J. D. GREEN. *Activity in single hippocampal pyramids.* Acta physiol. scand. 1960. *48.* 95—109. — Activity from single pyramidal cells of dorsal hippocampus has been studied in rabbits under light urethane anaesthesia or with *encéphale isolé.* Capillary microelectrodes were used for recording. Respiratory and cardiovascular movements of the brain were reduced by using a 'closed system'. Extracellular biphasic positive-negative spikes of large amplitude were frequently recorded. The shape of the extracellular spikes indicate that the soma spikes are usually not followed by excitation of the dendritic membrane. However, occasionally spikes with negative-positive potential sequence indicating somatofugal excitation of the dendrites, have been recorded after a burst of positive-negative spikes. This occurred in response to prolonged repetitive stimulation and in after-discharges. Spikes were normally not recorded in the dendritic layer. Repetitive stimulation, however, elicited sharp spikes in the apical dendrites increasing in number and amplitude with increasing stimulus strength and duration.

Action potentials and slow changes in membrane potential were commonly recorded with big attenuation but without other signs of serious injury.

Polarizing hippocampal neurones with minute currents in the order of 2×10^{-9}A through the recording microelectrode stimulated or inhibited that cell from which the activity was extracellularly recorded.

The hippocampus is an uncomplicated cortical structure with a relative uniform and simple lamination. These and other features make the Ammon's horn a suitable site for studies of cortical properties (see *e. g.* EULER, GREEN and RICCI 1958). This paper presents an analysis of the spontaneous activity in single hippocampal pyramids and a component analysis of spike potentials recorded extracellularly. A subsequent paper (EULER and GREEN 1960) deals with the activity patterns which can be elicited through afferent stimulation. A 'positive' wave with rapidly declining 'positive' spikes proved to be a pattern entity of possible significance for the 'brain waves' of the hippocampus. Although no successful intracellular records of this phenomenon were obtained, the experimental data presented in the present paper lend themselves to the conclusion that the spikes and the wave originate in the same neurone and that the phenomenon thus may be identified as the 'inactivating process' of GRANIT and PHILLIPS (1956).

Methods

Male and female rabbits averaging 3 kg in weight were used. The usual anaesthetic was urethane in doses of 1 g/kg; half administered intravenously and half subcutaneously. This produced a light but steady narcosis which was supplemented by ether during surgical procedures and by additional urethane as required. In order to evaluate the effects of urethane a few *encéphale isolé* preparations were also made.

For recording capillary microelectrodes, usually filled with 3M KCl, were used. In several experiments 4M NaCl was used instead. The micropipettes were drawn in a Shuster microforge and had when filled with 3M KCl solution, resistances of 5—30 MΩ. Cardiovascular and respiratory movements were reduced by using a 'closed' technique modified from GREEN and MACHNE (1955). A trephine hole with a diameter of approximately 7 mm was made with its centre at the coordinates P5 and L5 according to the stereotaxic system of SAWYER, EVERETT and GREEN (1954). The dura was carefully removed. A short brass cylinder with an outer diameter of 10 mm was put over the trephine hole. At the upper end the cylinder was cut across at right angles with the edges bevelled; the lower end was cut obliquely to fit the contour of the skull round the trephine hole when the tube was applied vertically. The brass tube was fastened tightly to the skull by means of dental 'sticky-Wax' (Klebewachs, 'Bayer', Leverkusen) and filled up with mineral oil. The microelectrode was inserted by a microdrive and the contact point with the surface of the cortex as observed from the noise level was read off on a dial indicator. The microelectrode was advanced another 500 or 1,000 μ and the upper end of the brass cylinder was sealed round the thick part of the microelectrode shaft with another kind of wax (Pinnacle blue inlay casting wax, 'Amalgamated Dental', London). A light glycerine application to the electrode allowed it to slide freely but tightly through the seal. The microelectrode was connected with a short chlorided silver wire to one of the grids of a differential cathode follower. The other grid and the silver-chloride reference electrode were earthed. The cathode follower output was led to a d. c. as well as a condenser-coupled amplifier; both permitted high gain amplification. The electrical activity was photographically recorded from two Cossor double beam cathode ray rubes provided with two time bases. A 15 MΩ electrode penetrating through 4 mm of brain tissue

(1 $\mu\mu$ F/mm, NASTUK and HODGKIN 1950) in addition to a probe capacitance of around 3 $\mu\mu$ F gives an input time constant of approximately 100 μsec. The amplitude of spikes recorded in hippocampus were thus attenuated by the recording system by 20—50 per cent depending on the shape of the spike and depth of the reacting site.

For stimulation concentric stainless steel electrodes were employed. They were guided stereotaxically to the desired coordinates through small drill holes in the skull. When the response showed that the stimulus site had been reached, the drill holes were sealed with the sticky wax. Two stimulators with delay circuits gave brief rectangular shocks (usually 0.8 msec duration) through isolating transformers either as single shocks or as tetanic bursts.

The direction of the microelectrode track was often marked by driving the electrode deep into the brain. Such enlarged recording tracks as well as stimulating sites were examined histologically on Nissl stained paraffin sections.

Results

As previously described by GREEN and MACHNE (1955), the penetration of the lateral ventricle by the microelectrode was often recognized by pulsation artefacts. The level of the alveus was thus found. This level is characterized by many brief spikes of low amplitude and mainly negative sign which give a characteristic sound in the loudspeaker. Three to five hundred microns below the ventricular artefact action potentials were recorded at very closely spaced intervals. This corresponds to the level of the pyramidal cell bodies. Four to six units could be recorded over a distance of 200—400 μ. Below this level usually no spikes were recorded for 500—800 μ. Then spikes were again recorded corresponding first to the layer of granule cells, and further down to pyramidal cells of the area CA_4.

When the microelectrode gradually approached an active neurone in the hippocampus, there suddenly appeared a biphasic initially positive spike. It was found preferable at this stage not to drive the micromanipulator any further, but to wait for the spontaneous increase in size, as described by GRANIT and PHILLIPS (1956) in their study of the cerebellar Purkinje cells. By further approach, practical after waiting a short time, the spike amplitude grew without any marked change in the relative amplitudes of its positive and negative phases. In this way it was possible to obtain spike amplitudes of ten to fifteen millivolts measured from positive to negative peak. The attenuation of these spikes has been discussed in Methods. Further attempt to approach the cells by turning the micromanipulator drive was usually followed by an increased rate of firing, a diminuation of the negative phase of the spike and a d. c. shift of a few millivolts. Penetration of the cell membrane was indicated by a big d. c. shift and a heavy discharge of monophasic positive spikes with firing rates up to 300/sec. The d. c. shifts thus obtained have been in the order of 24—40 mV. Occasionally membrane potentials above 50 mV were recorded. They were found at a depth corresponding to the pyramidal cells of area CA_4 where the largest hippocampal pyramidal cells

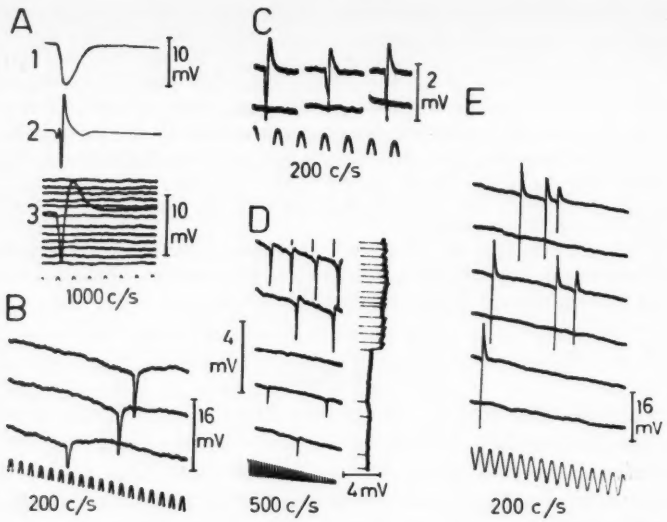

Fig. 1. Spike potentials from area CA_{1-2} of dorsal hippocampus of rabbit. Downward deflections denote positivity. *A*. 1. Monophasic positive spike. 2. The second derivative of the monophasic spike in *A* 1 is graphically constructed. 3. Biphasic positive-negative spike; 1 and 3 are d. c. records. *B*. Monophasic positive spikes on slow positive wave; d. c. record. *C*. Biphasic positive-negative spike with positive pre-potentials, the three spikes are obtained before, during and after repetitive stimulation to entorhinal cortex. During stimulation the spike has somewhat smaller amplitude and longer pre-potential-spike transition time; time constant 0.1 sec. *D*. Spikes of the monophasic positive type. Continuous trace: d. c. record; sweeps: time constant 0.1 sec. The step in d. c. level occurred at slight withdrawal of microelectrode. Note, close correlation between d. c. level and spike amplitude. *E*. Biphasic positive-negative spikes firing in bursts. Same spike as in diagram of Fig. 2 A—C. Time constant 1.0 sec.

are found. Sometimes big potential shifts were recorded which were not associated with any spike discharge. Similar findings have been reported from other nervous structures (*e. g.* MARTIN and BRANCH 1958).

In many cases only stimulation revealed that the electrode tip had almost reached contact with a cell membrane as indicated by the big amplitude of the provoked spike. Of course, many of these 'silent' neurones were never recognized until they were punctured, which most did not survive. A large number of the hippocampal pyramids were 'silent' for long periods of time in the unanaesthetized *encéphale isolé* preparations.

Sometimes 'silent' cells were fired by a movement of the microelectrode, probably by slight deformation of the neurone (ALANIS and MATTHEWS 1952). This treatment was sometimes observed to lower the threshold of the neurone to other stimuli. However, even when accelerated in this way, the spike

discharge could often be observed for a long time and its reaction to various stimuli was not qualitatively changed. At this time the spikes were often of 'normal' duration — 1.5 msec — which indicated that they had not undergone serious deterioration.

The spike potentials from the hippocampal pyramids have been recorded either as biphasic positive-negative or as almost monophasic positive spikes (Fig. 1). Spikes which were mainly negative relative to the remote reference electrode were rarely obtained in the hippocampus but more often found in the neocortex above it and especially in lateral geniculate body below the hippocampal formation (cf. TASAKI, POLLEY and ORREGO 1954).

Even those spikes which were 'caught' just above noise level were usually recorded with an initial positive phase.

The monophasic positive spike was very common in the pyramidal cell layer of the hippocampus. If not seriously deteriorated, it had approximately the same duration as the biphasic positive-negative ones, usually around 1.5 msec. The monophasic positive spikes resembled intracellularly recorded action potentials although of much lower amplitude and generally accompanied by only a small d. c. shift or no detectable shift whatever. Monophasic positive spikes could be kept for as long periods of time as could the biphasic spikes and they usually responded to orthodromic stimulation with the same discharge pattern as the biphasic ones. This shape, therefore, does not necessarily indicate a serious damage to the cell.

The biphasic positive-negative spike (*e. g.* Fig. 1 *A 3*) had usually a duration of around 1.5 msec. It always lacked a third positive component and the falling phase of the negative component was always slow. The shape of the biphasic spikes therefore did not display any evidence of conduction away from the electrode tip.

It is well known that amplitude decreases with accelerated firing largely because of increased inactivation (FRANKENHAEUSER 1952; HODGKIN and HUXLEY 1952). Changes in wave-form also occur. The biphasic extracellular positive-negative spike showed marked changes in the relative amplitudes of its positive and negative phases. This is shown in Fig. 1 *E* and in the diagrams of Fig. 2. The latter is obtained from records of single hippocampal pyramids characteristically discharging in short bursts. The positive and negative phases of the first spike in each burst are taken as references. The relative amplitudes of the positive and negative phases of the second and third spike in each burst are plotted against the intervals between first and second spike and between second and third one respectively. *A* shows the positive phases and *B* the negative ones, while the change in negative to positive ratio is given in *C*. Some interesting features are immediately evident from the diagrams. When preceded by only one spike, the positive phase is very little affected while the negative phase is markedly decreased for at least 20 msec. Two quickly preceding spikes, however, are followed by a greatly reduced

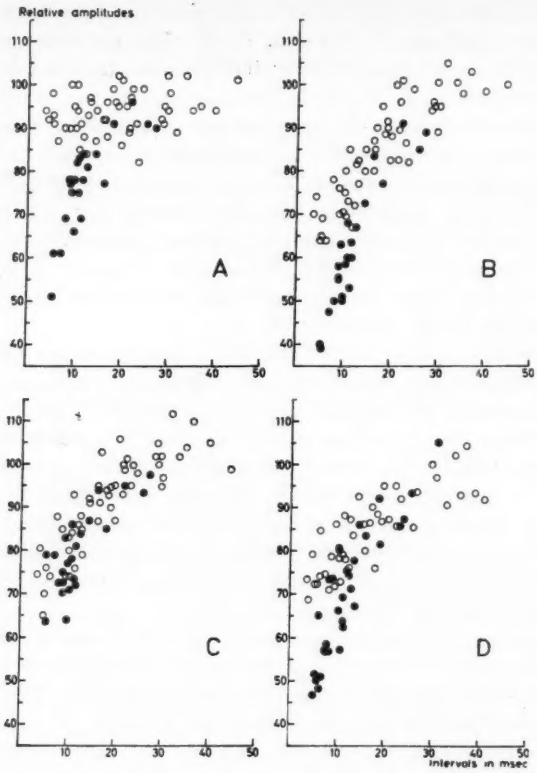

Fig. 2. Diagrams plotted from recorded activity of a neurone spontaneously firing in short bursts. Relative amplitudes of second spike (open circles) and third spike (filled circles) of a burst, as per cent of the amplitudes of the first spike, are plotted against the time intervals between first and second spike (open circles) and between second and third spike (filled circles). *A*—*C*. From one biphasic positive-negative spike. *A*. The positive phases, *B*. the negative phases and *C*. the negative/positive ratios. *D*. From monophasic positive spikes. Pyramidal cells of dorsal hippocampus of rabbit under urethane.

third spike. This reduction is most apparent in diagram *A* for the positive phase. The negative phase is affected just as much, since the plots of the ratio of negative to positive amplitudes versus spike intervals for the third spikes fit the course of the plots of the second spike. No measurable changes in the duration of the two phases have been detected. The changes in the membrane currents may therefore be assumed to be roughly proportional to the changes in amplitudes. The inward current thus seems to be reduced considerably more

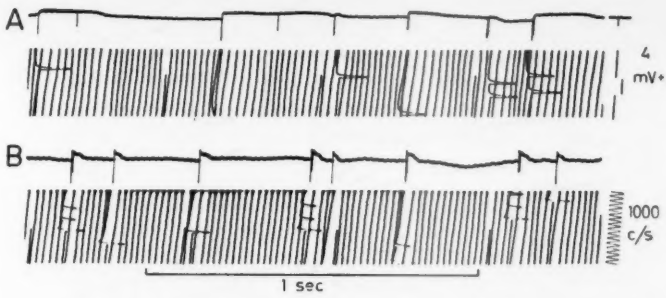

Fig. 3. From rabbit under urethane. *A.* and *B.* From two different pyramidal cells of dorsal hippocampus showing long lasting negative deflections with small spikes. Continuous record: d. c. Downward deflections denote positivity. Sweeps: time constant 0.1 sec. Voltage calibrations at *A.* and time calibrations at *B.* refer to both *A.* and *B.*

than the preceding outward current. In *D* of Fig. 2 the relative amplitudes of the second and third of a monophasic positive spike firing in bursts are similarly plotted. The monophasic positive spike, *D*, seems to correspond more closely to the negative component, *B* of the biphasic spike than to the positive component, *A*.

A phenomenon seen often enough in the hippocampal pyramidal cell layer to deserve mentioning is illustrated in Fig. 3. A spike is directly followed by a long lasting negative deflection as if the negative component of a biphasic spike were enormously prolonged. The prolonged negative deflection always starts with a spike or a burst of spikes and has often an even amplitude up to a rather abrupt termination. In other records it declines more slowly. The initiating spike is always markedly larger than the following spikes of the initial burst or those appearing later during the negative deflection. Most spikes occurring outside the negative deflection initiate one, while the smaller spikes, appearing during the negative deflection, do not add to it. This phenomenon of a long lasting step of depolarization following a spike has not been further analyzed. However, it has been observed to develop reversibly in response to passing a depolarizing current through the recording electrodes. The similarity to the prolonged action potential of the Purkinje heart muscle fibre (DRAPER and WEIDMANN 1951) is suggestive, but its nature remains obscure.

Prepotentials of various appearance were often present on the rising phase of the spike. They were as often evident on the biphasic as on the monophasic types of spike (see Fig. 1 and 4). Gradual irregular increase of outward current of long duration may precede the spike as in Fig. 1 *B* (*cf.* PHILLIPS 1956). In other records the prepotentials have a steeper rate of rise. Possibly they are signs of

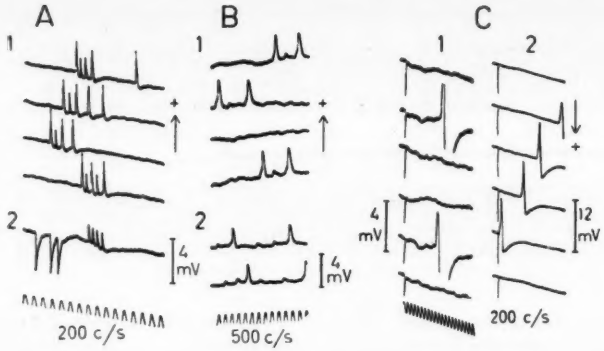

Fig. 4. Spike potentials from pyramidal cells of area CA_{1-2} in rabbit under urethane. In *A* and *B* upward deflections denote positivity; in *C* downward deflections denote positivity. *A*. 1, shows alternating big and small positive spikes in spontaneously appearing bursts. The small spike is roughly of same amplitude as the notch on the big spike. Stimulating septum with three shocks in rapid succession drove a short burst of impulses in that cell, however, only of the small amplitude (*A*. 2). d. c. records. *B*. Pre-potentials, some triggering spikes and some failing to do so. 1 and 2. Different neurones. Time constant 1.0 sec. *C*. Negative-positive spike of long duration. Stimulating septum at a rate of 15/sec revealed this spike which, at first, was driven by each second to each fourth shock (1). Later (2. at lower gain) it was firing more rapidly and independently of the shock stimuli. Time constant 1.0 sec. Note polarity, downward deflection denotes positivity.

excitatory post-synaptic potentials, but their durations were rather short when isolated from the spike. Fig. 1 *C* gives an example of lengthening of prepotential spike interval and decrease in spike amplitude when the cell is stimulated to increased firing rate. Some records show alternating big and small spikes which never summated and in which the small spike reached the same amplitude as the notch on the big spike (see Fig. 4 *A*), indicating that the spikes were generated in different parts of the neurone and that the big spike was triggered by the smaller spike in a manner similar to what has been described for various other types of neurones (see Eccles 1957, also for references). Sometimes prepotentials appeared to be subliminal for triggering a spike and appear alone alternating with successfully triggered spikes as in Fig. 4 *B*.

Effect of polarizing single pyramidal cells. It is well known that the hippocampal formation is very seizure susceptible. According to Jung (1949) the hippocampus has a lower threshold to electroshock than other cerebral structures, and Euler, Green and Ricci (1958) found evidence for ephaptic spread of excitation in the hippocampus. It was therefore considered worth-while to study the susceptibility of single hippocampal neurones to small polarizing currents. Minute currents were passed through the microelectrode so as to stimulate

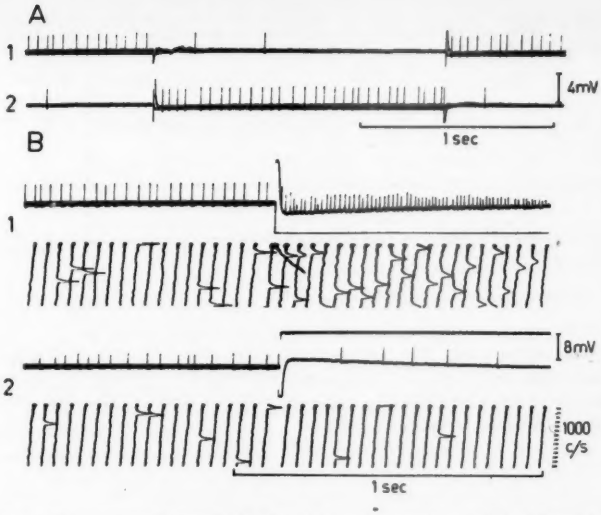

Fig. 5. Effects on hippocampal pyramids of rabbit under urethane elicited by polarizing currents through the microelectrode (KCl-filled). In *A*. 1. between the artefacts 3.6×10^{-9}A was passed inward through the electrode (tip negative). *A*. 2. Before the first and after the second artefact polarization with current of same strength and direction, in between no polarization. *B*. 1. 5.6×10^{-9}A is passed outward through the electrode (tip positive). In *B*. 2. current of the same strength is subsequently passed inward through the electrode (tip negative). Continuous and sweep records with time constant 0.1 sec. Positive spikes (see text).

that neurone from which the spike activity was recorded, using a technique similar to that of ARAKI and OTANI (1955). When a single hippocampal pyramidal cell had been approached to give a satisfactory spike, currents of either direction and different strengths were applied. As a rule currents directed outwards through the pipette electrode, *i. e.* making the electrode positive relative to the surrounding tissue, stimulated the neurone to increased firing rates. Currents of the opposite direction, rendering the electrode negative to the preparation, generally inhibited the discharge rates of the neurone. This was invariably the case when the monosynaptic positive type of spike was recorded.

Fig. 5 shows typical effects of polarizing currents. In *A 1* 3.6×10^{-9}A was passed inwards through the electrode between the artefacts and in *A 2* the same current was on before the first and after the second artefact with no polarization in between. In Fig. 5 *B* from another experiment the effects of 5.6×10^{-9}A are shown. In *B 1* current was passed outward through the electrode, making the latter more positive and depolarizing the cell. This

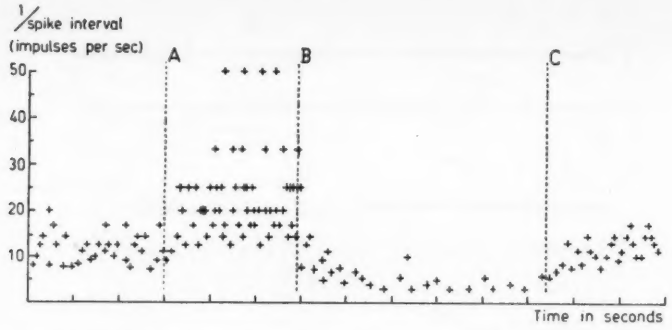

Fig. 6. Diagram showing the effect on discharge frequency of hippocampal pyramid of area CA_{1-2} to polarizing current of $1.8—10^{-9}$A outward through the electrode (tip positive) between *A* and *B* and in inward direction (tip negative) between *B* and *C*. See text for further description. Rabbit under urethane.

is obvious by the increased firing rate and the slowly increasing inactivation. Reversal of current after *B 1* readily restored the spike and later in *B 2* caused decreased firing rate and some increase in spike amplitude.

The described changes in discharge rates caused by the polarizing currents sometimes appeared more gradually, especially when applying weak currents as shown in the diagram of Fig. 6. Here the inverted value of the intervals between successive spikes (frequency) is plotted against time. The resting firing rate before polarization had a mean value of 11 impulses per second. Between the lines *A* and *B* the cell was polarized with a steady outward directed current of 1.8×10^{-9}A. The discharge rate increased and assumed a mean rate of 23 impulses per second. Reversing the direction of the polarizing current (between *B* and *C*) slowed the firing rate down to a mean of 4 impulses per second. When the polarizing current was switched off (at *C*) the discharge rate increased again to 11 impulses per second.

Fig. 7 gives another example of the effects of minute currents on neurones in the hippocampal pyramidal cell layer. *A* is control. During *B* and *C* a current of 5.4×10^{-9}A was passed outward through the electrode (electrode tip positive relative to brain). At artefact to the left in *D* the polarizing current was switched off. In this case polarization caused only a very slight increase in discharge rate. The figure is chosen because polarization of this neurone provoked patterns of slow wave of depolarization with rapidly deteriorating spikes on the rising phase which is believed to be identical with the 'inactivation process' of GRANIT and PHILLIPS (1956). The insets to the right of the continuous traces show the corresponding shapes of the spikes on an expanded time line. During polarization the negative phase is reduced.

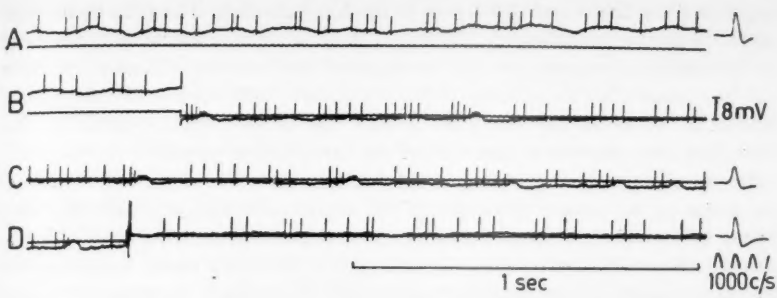

Fig. 7. From CA_1, of dorsal hippocampus of rabbit under light urethane showing effects of polarizing current. Upward deflections denote positivity. *A.* and beginning of *B.* control. Between artefacts in *B.* and *D.* 5.4×10^{-9}A was passed outward through the electrode (tip positive). After the artefact in *D.* no polarization. *A.*—*D.* Continuous d. c. record. To the right corresponding examples of single spikes on expanded time base to show the change in shape during polarization (*C*) as compared with controls to the right of *A.* and *D.*

Cathode make or anode brake excitations were not seen in response to such small currents and never obtained when the monophasic positive type of spike was recorded.

Discussion

Extracellularly recorded spike potentials of 10—15 mV amplitude are not unusual and have been obtained from other regions of cerebral and cerebellar cortex by *e. g.* GRANIT and PHILLIPS (1956) (Purkinje cells), and by PHILLIPS (1956) and MARTIN and BRANCH (1958) from Betz cells. Such large spikes must necessarily be recorded across a large recording resistance, as GRANIT and PHILLIPS pointed out. A marked increase in recording resistance apparently occurs as the cell is approached, judging by the noise level, and is measurable by the bridge circuit used here. It may involve an increase of several megohms (see also MATTHEWS, PHILLIPS and RUSHWORTH 1958).

It is presumed that potential signals recorded between a large remote 'reference' electrode and a small, well defined 'focal' extracellular electrode reflect currents of nervous origin flowing in the extraneuronal space. The current circuits are completed inside the neurones. Current flowing outward across the neurone membrane will be recorded as positivity and current flowing inward through the membrane at the site of active excitation will be recorded as negativity relative to the reference electrode. The amplitudes of the signals will represent current gradients. If an impulse is propagating along a uniform part of a neurone, the signal, recorded as described, will have a time course

approximating the second derivative of the corresponding change in membrane potential at the recording site (*cf.* Fig. 1 *A 2*). This time course will, however, be distorted by irregularities in the shape of the neurone. Conduction block will be apparent by the absence of the last positive deflection or of the negative deflection as well (depending on where the block occurs relative to the recording site) and by a slow fall of the terminating phase.

In our records the biphasic positive-negative spike has never approximated the shape of the second derivative of the action potential; especially the slow falling phase of the negative component and the absence of a third positive phase indicate that propagation of excitation from the soma membrane to the major parts of the dendritic membrane is blocked. It does not mean, however, that propagation out into the axon is also blocked, since the surface of excited axonal membrane must be very small in comparison to the area of unexcited membrane of the big dendritic bushes. This conclusion that propagation from the soma out into the dendrites normally is blocked confirms the previous results of EULER, GREEN and RICCI (1958) (*cf.* JUNG 1953, CREUTZFELDT, BAUMGARTNER and SCHOEN 1956, EULER and RICCI 1958, SVAETICHIN 1958). For the spinal motoneurone, however, FATT (1957) found evidence that an antidromic impulse propagates in somatofugal direction slowly along dendrites. A blockage may occur also in these dendrites before the terminals are reached (BARAKAN, DOWMAN and ECCLES 1949). However, sometimes when recording positive-negative spikes negative-positive potential sequences have been observed following a period of stimulation, indicating that excitation of soma membrane has been followed by excitation of a large area of dendritic membrane. The late positive phase of the negative-positive spikes showed variable amplitude and long duration. They have been observed after bursts of high firing rate, provoked by repetitive shocks on short tetani to septum, and in after discharges. This is a confirmation at the single neurone level of our earlier result (EULER, GREEN and RICCI 1958) that repetitive stimulation will excite more and more of the dendrites. This is believed to be in contrast with the hypothesis of PURPURA and GRUNDFEST (1956) that cortical dendrites should not be excitable by local electrical currents.

In the hippocampus it has been surprisingly common to record monophasic positive spikes. They have the same shape as the change in membrane potential, *i. e.* the action potential, although they are of much lower amplitude and generally are accompanied by only a small d. c. shift. There seem to be three main ways to explain this (TASAKI *et al.* 1954, MARTIN and BRANCH 1958). (i) The electrode is properly inside the neurone, which at this part has a very low membrane potential. (ii) The spike is recorded purely extracellularly, *i. e.* it reflects the membrane current which in these cases has only an outward direction under the electrode, probably because conduction of the impulse toward the electrode is blocked. (iii) The electrode is sealed into the membrane

or is in contact with an injured spot of the membrane which, however, is not big enough to let the membrane potential run down. Frequently there were no signs of deterioration. In this way the membrane potential and its rapid and slow variations would be recorded in an attenuated way.

Alternative (i) can immediately be excluded on the ground that monophasic spikes from more than one neurone have commonly been recorded simultaneously. We believe that the electrode cannot be inside more than one cell at a time.

In the majority of the monophasic positive spikes, there has been a much closer correlation between spike firing pattern and slow potential changes than ever has been seen when biphasic spikes were recorded. This high correlation, when it occurs, indicates that the slow potential changes seen together with the monophasic spikes are largely due to changes in membrane potential of the same neurone that generates the spikes. This is thus a support for the relevance of alternative (iii) *i. e.*, that the electrode is in contact with a small injured spot of the membrane through which the rapid and slow variations of the membrane potential are recorded in an attenuated way.

At high firing rates as in bursts of impulses the monophasic positive spike behaves as the negative phase of the biphasic spike *i. e.* as the phase of inward current. This is in harmony with alternative (iii) and does not agree with alternative (ii), according to which the monophasic positive spike would be a sign of outward current corresponding to the positive phase of the biphasic spike when conduction towards the electrode is blocked.

Stimulating the neurone from which the spike activity was recorded (ARAKI and OTANI 1955, FRANK and FUORTES 1956), cathode make excitations or anode brake excitations were not seen in response to the small currents used, and were never obtained when the monophasic positive types of spikes were recorded. This indicates that there was no excitable membrane between the electrode tip and the somaplasm. Since electrodes filled with 4 M NaCl had similar effects to those filled with 3 M KCl when polarizing the neurones the described effects are presumed not to be due to extrusion potassium ions.

In some cases two or three units were recorded simultaneously. In these cases the monophasic positive spikes of the biggest amplitude were the more sensitive to polarizing currents. When both a monophasic positive and a biphasic spike were recorded simultaneously, the monophasic spike was the more sensitive one to the currents tested, although the biphasic spike sometimes had the bigger amplitude. These results favour alternative (iii).

Concerning alternative (ii), one would assume that it would be possible to abolish a conduction block, if present, by changing the excitability of the neurone. In cases of biphasic spikes, changes in the shape of the spike were often seen with reduction or even abolition of the negative phase. This was caused by currents passing from the electrode outwards, and was usually fully reversible, indicating that the part of the neurone under the electrode

could be invaded or blocked to invasion by polarizing the cell in different directions. These effects of polarization which are gradual, progressive and reversible therefore closely resemble the changes in shape which occur in grouped biphasic spikes and are illustrated in Fig. 2. The monophasic positive spike which frequently terminates these bursts strongly suggests a reversible conduction block.

Since, on the other hand, we could not induce a negative phase by polarization in the majority of monophasic spikes they may be distinguished from the above. The implication is that a conduction block cannot be invoked to explain the shape of these spikes.

Thus, the evidence generally supports the concept that most monophasic positive spikes in the hippocampus reflected changes in membrane potential (*i. e.* proposition iii) rather than in membrane current only, (prop. ii) and that slow changes in the membrane potentials were similarly recorded in an attenuated way.

This conclusion has a very direct bearing on the phenomenon shown in Fig. 7 and further described in a succeeding paper (EULER and GREEN 1960) as a positive wave with rapidly deteriorating positive spikes riding on its rising phase. From the foregoing discussion it will be concluded that the spikes and the wave were recorded from the same neurone and that the spikes were inactivated (FRANKENHAUEUSER 1952, HODGKIN and HUXLEY 1952) by the slow, deeply depolarizing process recorded in the positive wave (GRANIT and PHILLIPS 1956).

The extreme sensitivity of the hippocampal pyramids to externally applied polarizing currents suggest however that electric fields occurring normally or under pathological conditions (EULER, GREEN and RICCI 1958) may be a factor of importance for the mechanism of synchronization of the neuronal activity in this very densely packed and uniformly oriented structure. Similar hypothesis have repeatedly been expressed in the past. However, quantitative studies of the voltage gradients across cortical structures have, as yet, not been made although TERZUOLO and BULLOCK (1956) have succeeded in making such an analysis on invertebrate neurones.

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Excitation, Inhibition and Rhythmical Activity in Hippocampal Pyramidal Cells in Rabbit

By

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Abstract

EULER, C. v. and J. D. GREEN. *Excitation, inhibition and rhythmical activity in hippocampal pyramidal cells in rabbit.* Acta physiol. scand. 1960. *48.* 110—125. — The activity pattern of single hippocampal pyramidal cells has been studied in rabbit under light urethane anaesthesia and with *encéphale isolé* in response to various stimuli. Deep depolarization leading to initial excitation followed by inactivation of spike generation has been found to be a commonly operating mechanism in the hippocampal pyramids. Such 'inactivation processes' have appeared spontaneously as well as in response to various stimuli. They may be driven with great precision as well as appearing rhythmically in after discharges. The 'inactivation process' was commonly followed by a hyper-polarization. Thus two different mechanisms of inhibition followed each other. Prolonged septal stimulation provoked trains of rhythmically recurring inactivation processes with bursts of declining spikes at the rising phase of each depolarizing wave.

Close similarities between the theta rhythm and rhythmically recurring inactivation processes are described and it is suggested that the former is built up by the latter synchronously occurring in several neurones. The inactivation process was also found to be a commonly occurring cellular event during seizure activity.

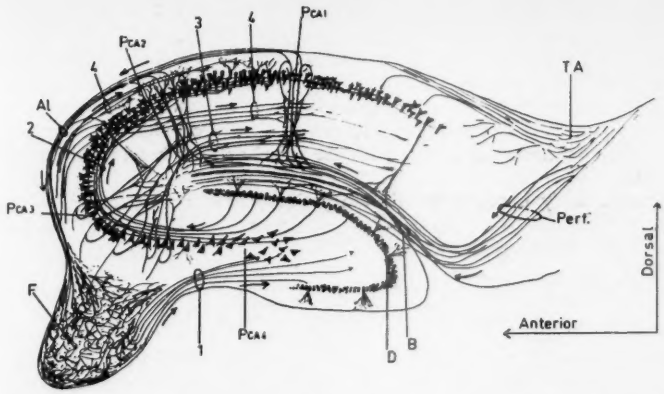

Fig. 1. Schema of dorsal hippocampus, sagittal section. Al — alveus. B — septal blood vessels which follow the pial cleft between the hippocampal cortex and fascia dentata — D. F — fimbria. P — hippocampal pyramidal cells of areas CA_{1-4}. TA — temporo-ammonic pathways. perf. — the perforating fibres of the latter, one portion of which ends among the terminals of the apical dendrites of hippocampal pyramids. The other portion terminates in the dentate fascia. 1—2 — pathway of GREEN and ADEY (1956). 3 — Schaffer collaterals of the big pyramids of CA_3. 4 — Fimbrial afferents ending among basal dendrites of CA_{1-3}.

In their analysis of hippocampal electrical activity in arousal GREEN and ARDUINI (1954) found that intact connections with septum and dorsal fornix were necessary to maintain theta rhythm. It is thus suggested that the axo-somatic connection of the pathway of GREEN and ADEY (1956) is of greater importance for the rhythmical activity of the hippocampus than are the axo-dendritic synaptic systems.

The present investigation of the effects of various afferent stimulation on the activity patterns of individual hippocampal pyramids, was undertaken to elucidate some of the mechanisms involved in physiological and pathological rhythmic discharges. Two different kinds of inhibitory processes as well as excitatory ones were found, to operate in some types of rhythmical discharges of bursts and after-bursts characteristic of hippocampal activity.

Four main fibre systems make synaptic contacts with the pyramidal cells of the dorsal hippocampus, three of these sets of synapses are mainly axo-dendritic and one is chiefly axo-somatic.

The temporo-ammonic pathway (see Fig. 1 TA) arises in the entorhinal cortex. One portion of the perforating fibres (Perf.) ends among the terminals of the apical dendrites of the pyramidal cells of areas CA_1 and CA_2 (LORENTE DE NÓ 1934, BLACKSTAD 1958). The other portion of the perforating fibres crosses the pial cleft (B) and terminates in the fascia dentata (D). Long axon collaterals (3) from the big pyramidal cells of CA_3 terminate among the apical dendrites of the CA_2 and CA_1. A third set of afferents comes from septum and dorsal fornix *via* a pathway analyzed by GREEN and

ADEY (1956). From the dorsal fornix and fimbria (F) the fibres (1) enter fascia dentata (D) and relay there onto granule cells. The axons of these (2) make synaptic contacts with or close to the cell bodies of the pyramids of area CA_2 and CA_3. The axo-somatic character of the latter set of synapses was confirmed by EULER, GREEN and RICCI (1958). A similar pathway is proposed by CRAGG and HAMLYN (1957) from the contralateral hippocampus. A fourth afferent system consists of commisural fibres from the contralateral side terminating among both the apical and the basal dendrites (BLACKSTAD 1956).

The present study concerns mainly the pathway from septum and the perforant pathway.

Methods

The methods and procedures used have been fully described in a preceding paper (EULER and GREEN 1960). Adult male and female rabbits were used under light urethane anaesthesia. Some *encéphale isolé* preparations were also used.

Capillary microelectrodes, usually filled with 3M KCl (in some cases with 4M NaCl), were used for recording. Cardiovascular and respiratory movements were reduced by using a closed system.

For stimulation concentric stainless steel electrodes were employed. They were guided stereotaxically to the desired coordinates according to the system of SAWYER, EVERETT and GREEN (1954). When the response showed that the stimulus site had been reached, the drill holes were sealed.

The track of the recording microelectrode was usually enlarged by driving the electrode deep into the brain. Such enlarged tracks as well as stimulating sites were examined histologically.

Results

Evoked responses in the dorsal hippocampus

Septal stimulation. GREEN and ADEY (1956) proposed a pathway by which impulses from the septum *via* the dorsal fornix enter the hilus of the fascia dentatus, relay onto the granule cells the axons of which again relay onto the hippocampal pyramids. This pathway was further studied by EULER *et al.*, (1958). They found an early sharp response of 1.5 msec latency in the fascia dentata (*cf.* Fig. 2) and a large response with latency of 4 or 6 msec and a duration usually of well over 20 msec in second order neurones among the pyramidal cells of area CA_2 of dorsal hippocampus.

In accordance with these results it was found possible by electrical stimulation of septum, dorsal fornix or ventral commissure to drive pyramidal cells of area CA_4 with a latency just below 1.5 msec (*cf.* Fig. 2) and cells of CA_{1-2} with latencies around 5 msec (*cf.* Fig. 4 *B*). Thus a unitary response could be evoked by each shock. However, in the rabbit under urethane anaesthesia the driving of pyramidal cells by septal stimulation usually required some potentiation either by repetitive stimulation, as shown in Fig. 3, or by using

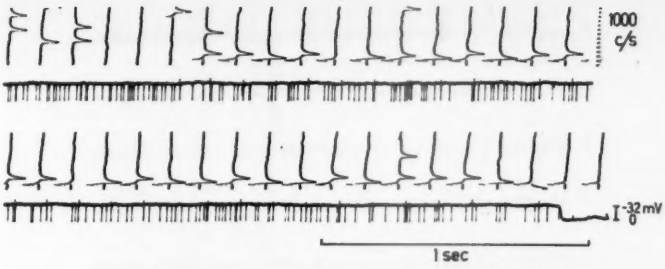

Fig. 2. Monophasic positive spike ('intracellular') from area CA_4 of rabbit under light urethane anaesthesia. From the seventh sweep shocks to dorsal fornix are driving the spike with latencies falling in two groups 1) too short to be measured with accuracy (offset of spike is fused with shock artefact), 2) 1.0—1.3 msec. Upper and lower records consecutive. Continuous trace: d. c. record; sweeps: time constant 1.0 sec.

a short tetanus. Sometimes repetitive firing was evoked in this way. The latency of 4—6 msec corresponds well with that of the main component of the evoked potential described in detail by EULER *et al.* (1958). Stimulating within these regions would activate not only the afferent pathway of GREEN and ARDUINI (1954) but also axons of the pyramidal cells of the hippocampus. Antidromic invasion was not, however, seen in the pyramidal cell bodies except in area CA_4 (fig. 2). The responses in area CA_4 may, however, indicate antidromic activation of these pyramids, which differ morphologically from those of other areas. This is in agreement with our previous finding (EULER *et al.*, 1958) that single shocks to dorsal fornix fail to invade the other pyramidal cells antidromically. Blockage of antidromic invasion has been described in various neurones (LLOYD 1943, BROCK, COMBS and ECCLES 1953, GRANIT 1955).

Fig. 2 shows 'intracellular' records at a depth corresponding to area CA_4 from a cell driven by stimulating septum. The latencies fall in two groups: 1) between 1.0—1.3 msec and 2) too short to be accurately measured probably just below 0.5 msec. The shortest latency indicates antidromic activation, while the longer latency is probably due to orthodromic excitation. It has also been common to drive a spike from septum with much longer latencies, around 20 msec (see Fig. 4 *B*). Whether these long latencies are due to long pathways or to rebound phenomena remains unknown.

In the rabbit inhibition has been as common in response to septal stimulation as excitation. Complete inhibition lasting up to one second without any initial excitation has frequently been obtained in response to single shocks to the septum. This is illustrated in Fig. 3. However, a repetition of the septal shocks, even at slow rates, soon revealed that an excitatory component was also activated. Thus, short bursts with short impulse intervals broke through

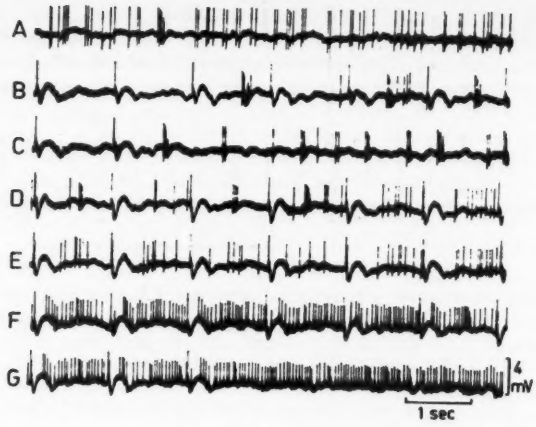

Fig. 3. From hippocampal pyramid of area CA_{1-2} of rabbit under urethane. Upward deflections denote positivity. *A*. 'Resting' activity. *B*—*G*. Shock stimuli to septum, interrupted for a short while in *C* and stopped in *G*. Time constant 1.0 sec. The loss of the negative component is associated with rapid firing, not death of the cell.

the inhibition. The excitatory component of the response was cumulative, as may be seen when comparing the 'resting' discharge in *A* with the activity provoked in *F* and *G*. Here, the initial period of inhibition is still evident though abbreviated. The short latency of the inhibition is obvious when seen against the background of high firing rate in the last part of *F* and beginning of *G*. In Fig. 4 *A*, again, inhibition and excitation were both in play, although the balance was somewhat different from that in Fig. 3. From Fig. 4 *A*, it is further evident that the excitation was cut short by a late inhibition.

'*Inactivation process*'. In the rabbit a common response to septal stimulation has been a process of inhibition preceded by brief initial excitation similar to that described by GRANIT and PHILLIPS (1956) as inhibition by cathodal depression or inactivation (FRANKENHAEUSER 1952, HODGKIN and HUXLEY 1952), and which they called the 'inactivation process'. The character of deep depolarization is apparent in records where monophasic positive spikes are obtained. Fig. 5 and 6 show waves of different time course with declining spikes. Such responses of waves with rapidly deteriorating spikes have been provoked by selective stimulation of that neurone from which the spikes are recorded by passing a small current outward through the recording electrode. This shows that the wave and the declining spikes are generated within the same neurone. Further evidence is given elsewhere (EULER and GREEN 1960).

Fig. 5 *A* shows an 'inactivation process' in response to short tetanic stimuli to septum. Since this neurone was otherwise 'silent' the inhibition by inactiva-

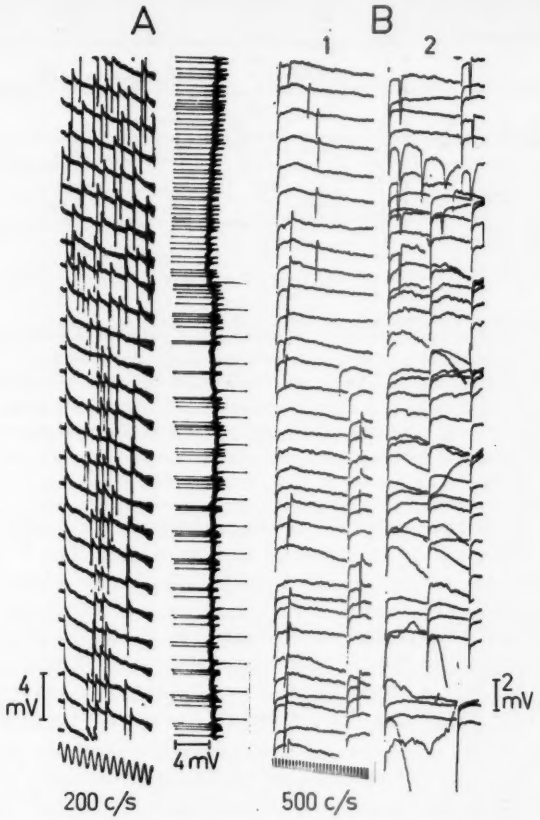

Fig. 4. Biphasic positive-negative spikes from pyramidal cells of area CA_{1-3} of rabbit under urethane. Repetitive shocks to septum. To be read downwards and from left to right. Downward positivity in sweeps *A*. After a few shocks excitation and inhibition has reached a balance which then is maintained. Excitation is obvious from the significant increase in the relative number of short impulse intervals and appearance of shorter intervals during stimulation than ever occurred before stimulation. Continuous trace: d. c. record, sweeps: 0.1 sec time constant. B. 1 and 2 are consecutive. Spikes are driven alternately with latencies of 4—6 msec and around 20 msec. In the middle of B. 1 the rate of stimulation was doubled (two shock artefacts on each sweep) and during B. 2 it was doubled again (three artefacts on each sweep, the fourth is falling in the sweep interval). Just before the last increase in rate of stimulation seizure activity developed (2; 5th sweep from top), which leads to complete block of driving the spike. Time constant 0.1 sec.

tion was not obvious, only the short burst of declining spikes excited before inactivation was complete. *B* of the same figure shows series of undriven

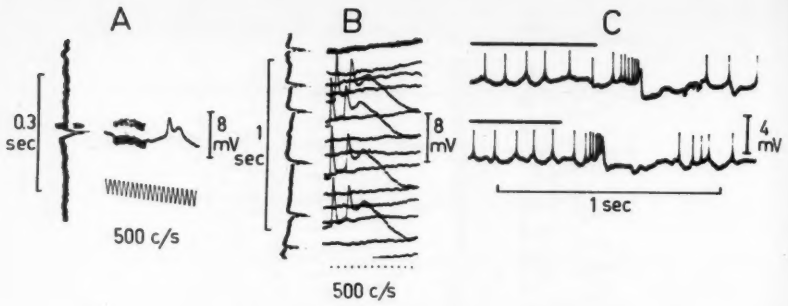

Fig. 5. 'Inactivation processes' from rabbit hippocampus. Upward deflections denote positivity. *A*. Response from pyramid of area CA_4 to a short tetanic stimulus to septum. Continuous and sweep: time constant 0.1 sec. *B*. (To be read from below upwards) 'Inactivation processes' from pyramid of area CA_{1-2} during short after discharge. Not driven. Continuous trace: d. c.. Sweep: 0.1 sec time constant. *C*. Two examples of off-responses to light flash from pyramid of area CA_{1-2}. d. c. records.

spontaneous inactivation processes during a short seizure discharge. *C* shows 'inactivation processes' as off-responses to light flashes. The transition from deep depolarization to long lasting hyperpolarization is evident.

It has frequently been observed that the 'inactivation process' is followed by an overshooting backswing to opposite polarity which strongly suggests that the hyperdepolarization in these cases is followed by a phase of hyperpolarization, usually of longer duration. During this latter period generation of impulses is also inhibited. This is illustrated in Fig. 5 *C*, 6 *A* and *B*.

Stimulation of the septum repetitively with single shocks or high frequency tetani often provoked a long series of 'inactivation processes' outlasting the stimulus and gradually assuming a very regular rhythm of 3 to 6 per sec (see Fig. 6). Single shocks to septum interposed in such a regular sequence of 'inactivation processes' elicited, as illustrated in Fig. 6 *B*, an 'inactivation process' with a short latency independent of where in the rhythm the shock fell. This driven response reset the rhythm so that the preceding sequence was broken and the triggered response started a new sequence of rhythmically recurring 'inactivation processes'. Tetanic stimulation to septum in the middle of a train of rhythmically appearing 'inactivation processes' usually cuts the train off. The regular rhythm reappears gradually after the end of the stimulus, often reinforced with bigger amplitudes and increased regularity.

In true extracellular records, *i. e.* when biphasic spikes of short duration are recorded as in Fig. 7, 'inactivation processes' may not be so easily recognized. However, a train or a burst of impulses may be cut short by a negative deflection. Sometimes this negative deflection may be followed by a positive deflection which indicates that the region of excitation was propagating

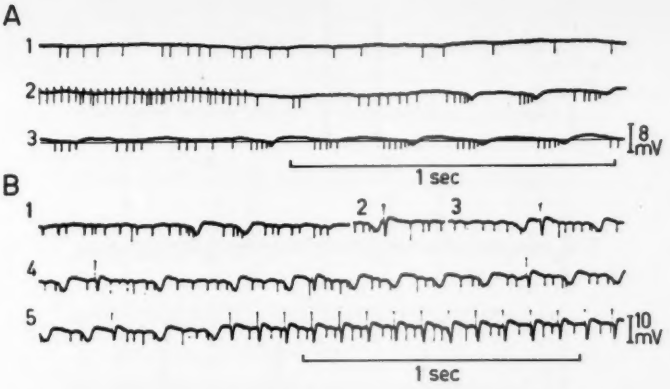

Fig. 6. Rhythmically recurring 'inactivation processes' in pyramidal cells of area CA_1 of rabbit under urethane. Downward deflections denote positivity. d. c. records. *A.* Before (1), during and after (2 and 3) repetitive stimulation to septum. 2 and 3 are consecutive. *B.* 1: control, 2 and 3 are samples from record of repetitive stimulation to septum with short tetani (3 shocks in 5 msec). This stimulation evoked rhythmically recurring inactivation processes seen in 4 and beginning at 5. This rhythm was interrupted and reset by single shocks to septum (4 and 5).

away from the electrode. The amplitude of this positive deflection varied considerably in records from the same cell (see Fig. 7) suggesting a variation of the membrane area engaged. The falling phase of this positive deflection is much slower than that of the same spike (*cf.* EULER and GREEN 1960, their Fig. 4 *C*).

The theta rhythm. In their paper on hippocampal electrical activity in arousal, GREEN and ARDUINI (1954) analyzed the theta pattern of slow waves in the rabbit hippocampus as a response to various afferent stimuli, originally observed by JUNG and KORNMÜLLER (1938) and GERARD, MARSHALL and SAUL (1936) in the cat. GREEN and ARDUINI found that this type of hippocampal response is mediated by the precommisural fornix, septum and the dorsal fornix. Accordingly they found that this response was prevented by septal lesions or section of the dorsal fornix and that it could be elicited by direct stimulation by these structures. In the experiments reported in the present paper on rabbits under urethane and with *encéphale isolé*, the theta response of the hippocampus was often obtained to various afferent stimuli: flashes of light, noise, twisting the pinna, etc., as well as to electrical stimulation of the septum.

In agreement with GREEN and MACHNE (1955) and ARDUINI and POMPEIANO (1956), some correlation between spike discharge pattern and theta rhythm was often found. This is very obvious in Fig. 8, where the spikes

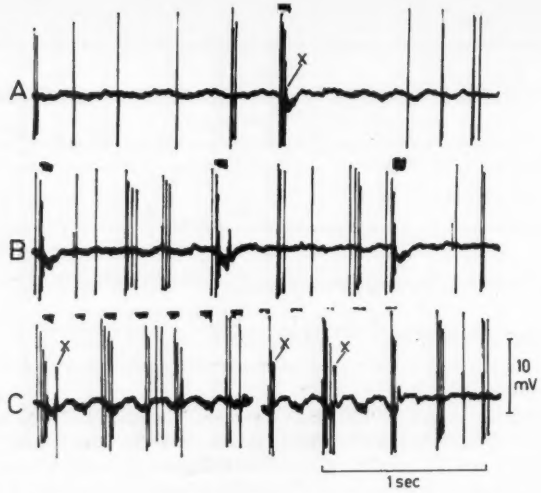

Fig. 7. Biphasic positive-negative spike from pyramid of area CA_{1-2} of dorsal hippocampus of rabbit under urethane. Upward deflections denote positivity. Time constant 1.0 sec. Short tetanic stimuli to septum indicated by shadows at the upper border of each record, one in *A.*, three in *B.* and twelve in *C.* The tetani evoke negative deflections with inactivation and now and then a negative-positive spike marked with ×.

regularly fall on the positive summit of the potential waves. In other records the relation between spikes and the phase of the slow activity was not precise, although there was an obvious modulation of spike frequency following the theta rhythm. Occasionally it has been observed that a neurone may discharge impulses apparently uninfluenced by simultaneously occurring theta activity in neighbouring neurones.

Single shocks to the septum falling in the regular theta rhythm stopped activity and reset the rhythm in the way described above for the rhythmically reappearing 'inactivation process'. Similarly, repetitive shocks to the septum may drive the theta rhythm to follow the stimulation frequency within narrow limits, as is shown in Fig. 8 *C—F.* Tetanic stimulation of the septum, as in *J—L*, generally causes inhibition both of the slow waves and of spike activity. This inhibition continues after the cessation of the stimulus, and its duration is roughly proportional to the duration of the tetanic stimulus. Gradually the rhythmical slow wave activity is resumed in a way similar to what is described above for the rhythmically recurring 'inactivation processes'. The inhibition and the cumulative rebound facilitation of shock responses to septum was illustrated in Fig. 3.

Afferent stimulation. The most prominent response to various afferent stimuli

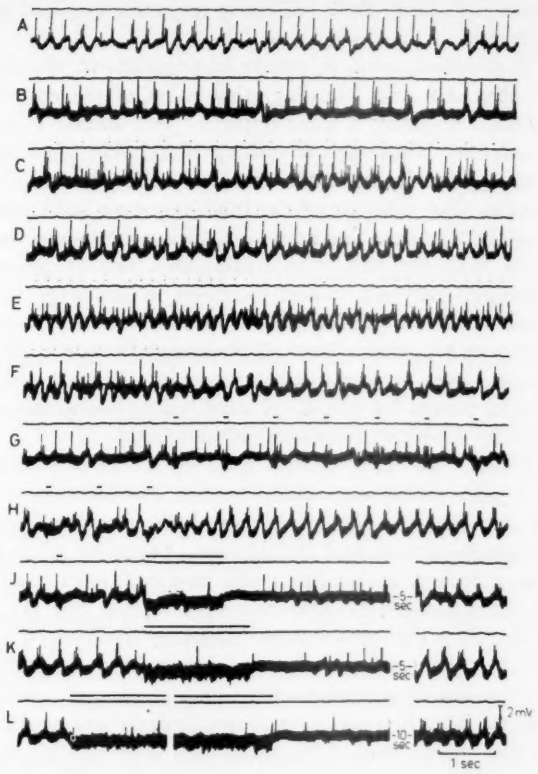

Fig. 8. From area CA_{1-2} of dorsal hippocampus of rabbit under urethane. Upward deflections denote positivity. *A*. Control. *B—F*. Single shocks to septum with increasing repetition rate disturbing the theta rhythm at the lower frequencies in *B*. and *C*. and driving the rhythm in *E*. and beginning of *F*. In *G*. and *H*. septum is stimulated with short tetanic stimuli inhibiting the theta rhythm which reappears reinforced at the cessation of stimulation. *J—L*. Same effect using long tetanic stimulation to septum. Stimulus duration in *L*. 5.5 sec. Time constant 1.0 sec. Shock artefacts indicated by dots above upper trace.

was the appearance of theta waves (GREEN and ARDUINI 1954). In the single unit records the results of GREEN and MACHNE (1955) were confirmed. They described short bursts of impulses as being a common response to light flashes. In addition to this observation it was now found that these short bursts were carried on the rising phase of a wave of deep depolarization, *i. e.* an inactivation process. Such responses have been obtained both as on- and off-responses to flashes of light and to twisting the pinna. Fig. 5 *C* shows an example of an

off-response to a light flash. Pure inhibitory responses to visual stimuli have also been observed.

Stimulation of entorhinal cortex. A variety of responses in the dorsal hippocampus have been observed following stimulation of the entorhinal cortex on the ipsilateral side. Many of these responses were similar to those elicited by septal stimulation. Thus high frequency stimulation often provoked inhibition of 'resting' activity in single units which lasted considerably longer than the stimulus. The duration of the inhibition correlated positively with the duration of the stimulus. The inhibition during the tetanus was generally less pronounced than the post tetanic inhibition. During prolonged tetani the spike sometimes reappeared and increased in discharge rate, but was again inhibited when stimulation was stopped. Rebound increase of the discharge frequency was often observed sometimes broken up in rhythmical activity by slow waves or 'inactivation processes'. Theta waves have only occasionally been evoked by entorhinal stimulation, but seizure activity was often provoked by repetitive entorhinal stimulation.

It has often been possible to drive a spike by shocks to entorhinal cortex, although some potentiation was usually needed. The latencies for the driven spikes recorded in CA_2 and CA_1 of the dorsal hippocampus have fallen into two main groups, 4—6 msec and 10—12 msec. Latencies of less than 2 msec have also been recorded. Spikes that could be driven from entorhinal cortex were usually not influenced by stimulating the septum and *vice versa*. Spikes that were driven from the septum were commonly uinfluenced by entorhinal stimulation. Only once a spike was found which could be driven from both the septum and from the entorhinal cortex.

Seizure activity. The hippocampus is particularly susceptible to seizure activity, which is readily evoked by mechanical and electrical stimulation of Ammon's horn and its afferent connections (JUNG 1949, KAADA 1951, GREEN and SHIMAMOTO 1953, CREUTZFELDT 1956, GREEN and ADEY 1956). EULER *et al.* (1958) investigated the role of the apical dendrites in convulsive electric activity in the hippocampus. They found that somatofugal conduction in the dendrites was normally blocked when the hippocampal pyramids were synaptically excited by single shock stimuli to the dorsal fornix. Repetitive stimulation, however, activated more and more of the dendrites until finally most of the dendritic trees were actively engaged. At this moment seizure activity broke through.

In accordance with these results it was observed that spikes were very rarely obtained in the dendritic layer. However, repetitive stimulation of the septum or opposite hippocampus frequently provoked spikes of negative polarity in the dendritic layer. They were usually of rather short duration, often 4—6 msec and mostly of big amplitude. An example is illustrated in Fig. 9. Their amplitude and duration varied with stimulation strength and frequency. These spikes were present throughout large dendritic areas, and

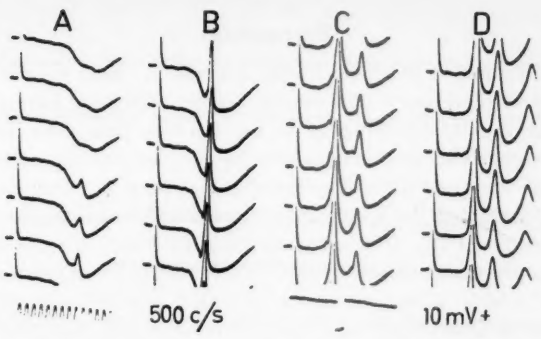

Fig. 9. Potentials evoked by repetitive septal shocks recorded from the dendritic layer of area CA_{1-2} of dorsal hippocampus in rabbit under urethane. Downward deflections denote positivity. Continuous stimulation at 10/sec. Approximately 3 sec intervals between the records *A. B. C.* and *D.* Negative spikes are growing from an increasing positive wave. As stimulation continues more spikes appear and grow to bigger amplitudes. Time constant 1.0 sec.

they were not readily lost by moving the electrode despite amplitudes over 20 mV. Since single unit spikes were very rarely recorded among the apical dendrites, it can be concluded that these driven spikes which could be obtained only on repetitive stimulation were due to synchronous activity in the dendrites of several neurones. These spikes were usually superimposed on a positive wave and increased in number with the duration, frequency and intensity of the repetitive stimulus. If stimulation was stopped when such a state had developed, a self-sustained after-discharge replaced the driven activity and developed into new patterns, often in an irregular, unpredictable way, until it stopped in the 'isoelectric' phase of the seizure. If stimulation continued long enough, the seizure discharge 'broke through' the driven dendritic activity. During the following seizure discharge the shocks which had provoked it did not elicit any responses independently of where in the discharge pattern the shocks happened to fall (Green and Adey 1956). During the seizure activity the neurones seemed to be continuously blocked in some way to synaptic activation *via* the ordinary pathways. This was true also for the first order synapse in the dentate gyrus.

At the soma layer of the hippocampus the single cell activity during seizure was often characterized by series of 'inactivation processes' appearing in more or less regular rhythms. Such self-sustained discharges of 'inactivation processes' during seizure activity could usually not be influenced by single shocks or tetanic stimulation to the septum nor to the entorhinal cortex.

Spikes with a negative-positive sequence were frequently recognized in the seizure-pattern of the soma layer. The late positive phase of these negative-positive spikes was usually of long duration and of variable amplitude.

Discussion

The identification of the 'inactivation process' is based on the conclusions drawn in a preceding paper (EULER and GREEN 1960) that both the 'positive' wave and the rapidly declining spikes on its rising phase are recorded from the same neurone. This view is supported by the close correspondence between the wave and the generation of spikes that has been so commonly recorded. We thus conclude that the spikes are triggered and subsequently 'inactivated' (FRANKENHAEUSER 1952, HODGKIN and HUXLEY 1952) by the slow, deeply depolarizing process recorded in the positive wave. Similarly the negative going wave which often has been recorded immediately following the positive wave is interpreted as a process of increased membrane potential, *i. e.* a hyperpolarization. There is inhibition of impulse generation also during this latter phase. Thus, two processes of inhibition follow each other; at first the impulse discharge is cut off by means of inactivation, whereafter the inhibition is maintained by hyperpolarization.

In the hippocampus the initial period of excitation at the foot of gradual depolarizing waves has been as prominent as the following inhibition by 'inactivation'. Inactivation processes thus modify the discharge pattern so as to produce short high frequency bursts of spikes characteristic for the activity of several central structures. BURNS (1954, 1955, 1958) has proposed a mechanism for after-bursts based on the assumption of 'differential repolarization'. Since, however, dendrites are not necessarily invaded by the impulse (EULER, GREEN and RICCI 1958, EULER and RICCI 1958), this hypothesis can only be valid for circumstances under which the dendrites or significant parts of them are actively depolarized. Invasion of the dendritic tree from the soma seems to be favoured by the deep, long lasting depolarization leading to inactivation, *i. e.* the inactivation process. Thus, it does not seem likely that bursts of impulses are due to a mechanism similar to that suggested by BURNS. However, the rhythmic recurrence of 'inactivation processes' with their initial bursts and subsequent hyperpolarizations might be favoured by such a mechanism.

The results have shown close similarities between these rhythmically recurring waves and the theta activity (see Fig. 10). The theta rhythm, however, is best recorded with comparatively high gain and under circumstances favouring a few neurones being recorded simultaneously, *i. e.* when the electrode has not approached any one neurone so closely as to give high recording resistance and, consequently, high amplitude spikes. The inactivation processes, on the other hand, are not easily identified unless the conditions are optimal for selective recording of single neurones and are preferentially studied in the semi-intracellular records, *i. e.* when monophasic positive spikes were recorded.

It is thus suggested that the theta rhythm is due to synchronously occurring

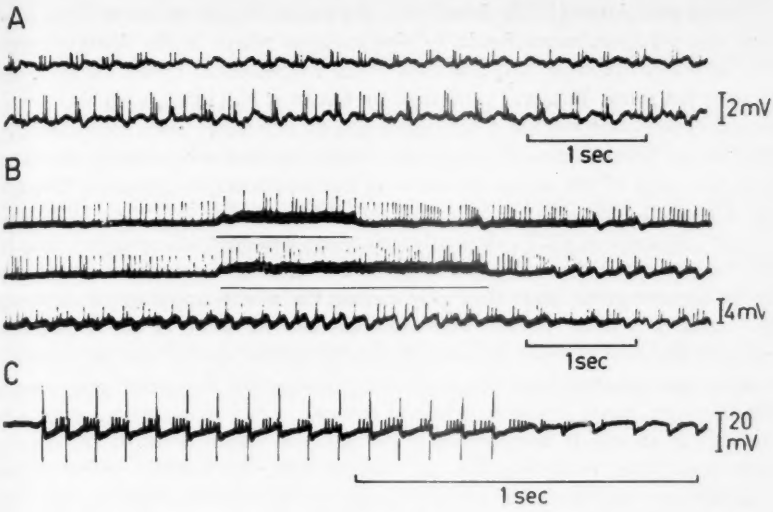

Fig. 10. From dorsal hippocampus of rabbit under urethane. d. c. records. Upward deflections denote positivity. To show similarity between theta rhythm, *A.* and inactivation processes, *B.* and *C.* In *B.* tetanic stimulation to septum, marked with horizontal lines, provoked rhythmically recurring inactivation processes. *C.* Shocks to contralateral hippocampus evoking rapid depolarization followed by long lasting hyperpolarization and rebound of slow depolarization with burst of spikes. Note, after discharge with hyperpolarization and long lasting depolarization with inactivation.

'inactivation processes' in many neurones. Similarities were found concerning the following features. (i) The two types of rhythm have usually shown the same range of frequencies. (ii) Repetitive stimulation of septum is a potent way of provoking both recurring 'inactivation processes' and theta rhythm. (iii) Afferent stimulation may provoke both types of rhythms. (iv) Entorhinal stimulation has only occasionally provoked theta rhythm and rhythmically occurring 'inactivation processes'. (v) Both types of rhythm may be interrupted and reset by single shocks to the septum. (vi) Tetanic stimulation to septum has caused prolonged inhibition of both types of rhythmical activities.

Green and Arduini (1954) found that intact connections from the septum were a pre-requisite for obtaining the theta pattern by afferent stimulation and further that those structures from which the theta rhythm could be provoked by electrical stimulation project to the septum. However, the septum itself was not the electrical source of the rhythmical activity corresponding to the theta waves. This rhythm is thus generated in the hippocampal formation under the pressure of afferent impulses from septum and precommissural fornix.

GREEN and ADEY (1956) found that the main afferent pathway from septum and precommissural fornix to hippocampus relays in the dentate gyrus and activates the areas CA_3 and CA_2 of the hippocampal cortex *via* synapses close to the soma. This was confirmed by EULER *et al.* (1958), who also found that the dendrites were not actively engaged when the soma was fired by this route. Stimulation of entorhinal cortex, on the other hand, activates terminal parts of the apical dendrites of the hippocampal pyramids through the perforant path of CAJAL (see CAJAL 1911, pp. 768—775, or 1955, pp. 89—99, BLACKSTAD 1958). Activation *via* these axo-dendritic synapses usually did not provoke a theta rhythm. A portion of these perforant fibres terminates in the dentate gyrus. Here they may contact the septal-hippocampal pathway of GREEN and ADEY (1956). This may account for some of the similarities between response to septal and entorhinal stimulation and for why occasionally a theta pattern has been obtained after stimulating the entorhinal cortex. On the other hand, it was surprisingly unusual to find hippocampal pyramids that could be driven from both septum and entorhinal cortex. If the usually postulated types of termination are correct then, the cortical waves in the hippocampus are thus maintained through synapses of axo-somatic character and not by axo-dendritic ones.

It has been argued above that the 'inactivation processes' and related hyperpolarizations may be the cellular events which build up the theta waves. The mechanisms of synchronization, remain unexplained. It has often been observed that neighbouring neurones discharge impulses fairly synchronously even in the absence of theta waves or of pathological activity. 'Inactivation processes' have also been recognized as single neurone activity in convulsive synchronous discharges.

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5-Hydroxytryptamine in Burns

By

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Abstract

JOHANSSON, S.-A. *5-hydroxytryptamine in burns.* Acta physiol. scand. 1960. *48.* 126—132. — The relative amounts of 5-hydroxytryptamine (5-HT, serotonin) in skin, blood, and urine after burn injuries have been determined spectrophotofluorometrically after extraction into n-butanol. Increased amounts of 5-HT in rat skin and a decrease in the number of circulating platelets accompanied by a decrease in the total amount of 5-HT in rabbit whole blood were found after burn injuries. In five human patients with severe burns an increase of the urinary excretion of the 5-HT metabolite, 5-hydroxyindole-acetic acid (5-HIAA) was found, which seems to indicate an increase in the endogen metabolism of 5-HT. It is suggested that blood platelets inactivate free 5-HT and that heparin can inhibit the liberation of 5-HT from the blood platelets in burn injuries and anaphylactic reactions.

In experimental burns in animals immediate removal of the burned area prevents the onset of symptoms such as delirium, vomiting, bloody diarrhoea, and circulatory failure. If the burned area is transplanted into a normal animal, these symptoms will appear in that animal (ROSENTHAL 1937). PRINZMETAL and HECHTER (1944) have shown that a kind of shock syndrome, that develops after burn injuries on the hind legs of rats caused by water at 75° C and 100° C during 60 sec and 2—3 min, respectively, depend to a less degree on the loss of fluid than on toxic or nervous actions. These facts suggest that substances are liberated, which are of importance for the development of a complex illness — the burn injury.

Burn injuries are nearly always followed by oedema. Little is known of its formation. It is interesting to note, however, that subcutaneous administration

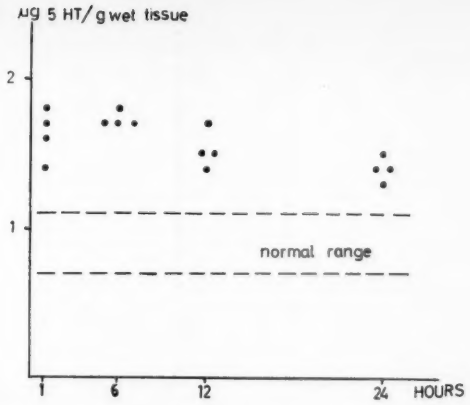

Fig. 1. 5-HT content of rat abdominal skin during the first 24 hours after the injury.

of 5-HT causes a marked increase in capillary permeability (ROWLEY and BENDITT 1956). 5-HT is about 200 times as effective as histamine in producing oedema of the rat's paw (ROWLEY and BENDITT 1956). The oedema producing effect of protein or dextran in rats is inhibited if the animals have been pretreated with the 5-HT liberating substance, reserpine (WEST 1957). 5-HT also increases the permeability of the beet root cell membrane to water soluble pigments (PICCLES and SUTCLIFFE 1955). Along similar lines PICCLES (1956) found that pretreatment of erythrocytes with 5-HT accelerates their subsequent hypotonic hemolysis. 5-HT thus seems to produce alterations in the permeability of the cell membrane.

The object of this investigation is to study the relative amounts of 5-HT in skin, blood and urine after burn injuries.

Experimental

Rat experiments. Of the mammalian species only the rat, as far as known has relatively large quantities of 5-HT in its skin. Thermal burns were therefore produced with water at about 65° C and with hot iron (area about 3 cm^2) the temperature of which was adjusted by a thermostat to 65° C on the shaved abdominal skin of 16 nembutal anesthetized albino rats of both sexes weighing 150—200 g. The skin was burned during 60 sec. (about 15 per cent deep third degree burn). The rats were killed by decapitation at 1, 6, 12 and 24 hours, respectively, after the injury. Eight nembutal anesthetized albino rats served as controls. The full thickness of the skin and the subcutaneous fat were taken in the burned areas and were extracted for their content of 5-HT and other indole compounds.

Rabbit experiments. Rabbit were chosen for these experiments because they have a high content of 5-HT in the blood. Rabbits of both sexes weighing 2.5—3 kg were used. They were anesthetized with nembutal. About 20 per cent deep third degree burns

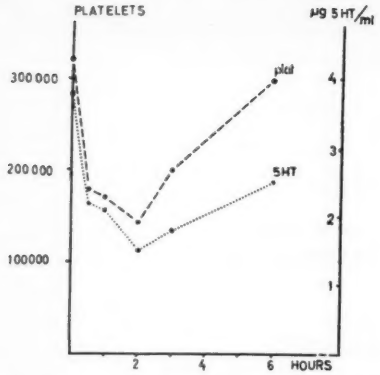

Fig. 2.

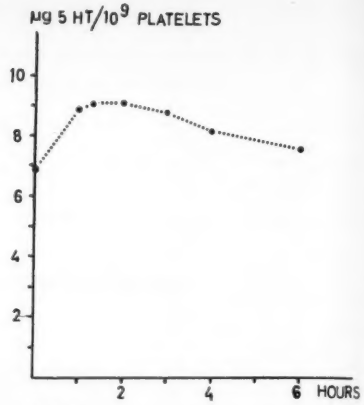

Fig. 3.

Fig. 2. 5-HT in whole blood and platelet counts during the first 6 hours after the burn injury.
Fig. 3. The 5-HT content of 10^9 platelets during the first 6 hours after the burn injury.

were produced with hot water (65° C) in 10 shaved animals. The technique was the same as in the rat experiments.

Ten ml blood samples from the rabbits were drawn by heart puncture at two and one hour before and at 30 min, 1, 2, 3, and 6 hours after the injury. Nine volumes of blood was collected into centrifuge tubes containing 1 volume of 3.8 per cent trisodium citrate ($Na_3C_6H_5O_7 \times 2\ H_2O$). Silicon treated vessels were used throughout. Platelets and platelet deficient plasma were prepared as earlier described (Nilsson *et al.* 1957). Platelet counts were made by the method of Kristensson. The 5-HT content was determined in whole blood, platelets, and platelet deficient plasma.

Clinical Material. The clinical material consists of five patients with severe burns. The clinical data of these patients and others will be published later together with Birke and Liljedahl in Acta chir. scand.

The urinary excretion of 5-HIAA was estimated from 24 hours samples.

Human platelets were incubated at 37° C in plasma or 0.9 per cent saline with 2 µg per ml of 5-HT. The platelets were then washed 3 times with 0.9 per cent saline and their content of 5-HT was measured. Platelet counts were made before the incubation and after by the method of Kristensson.

Chemical analyses. The 5-HT concentrations in blood, blisters and tissues were estimated spectrophotofluorometrically after extractions into purified butanol according to Udenfriend, Weissbach and Clark (1955). The 5-HIAA analyses in urine were carried out spectrophotometrically after extraction with ether according to Udenfriend, Weissbach, and Clark (1955) and to Macfarlane *et al.* 1956. Paper chromatography of the tryptophane metabolites was carried out according to Dalgliesh (1956).

Results

Rat results. The 5-HT content of abdominal skin after thermal burns at different times after the injury is shown in Fig. 1. The extractable amounts of 5-HT increase as early as within one hour after the injury and remain on a

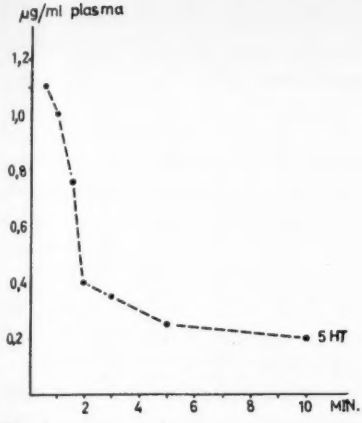

Fig. 4.

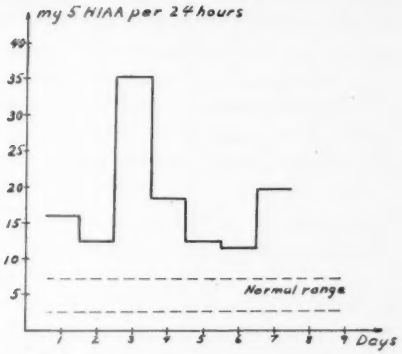

Fig. 5.

Fig. 4. Plasma content of 5-HT following intravenous injections of the amine.
Fig. 5. Urinary excretion of 5-HIAA during the first eight days after the burn accident in one patient.

high level for at least 24 hours. In the paper-chromatographic analyses no 5-HIAA or other indole compounds were found in the extracts.

Rabbit results. The rabbit, contrary to the rat, has no appreciable amounts of 5-HT in the skin, but rabbit blood is extremely rich in 5-HT. Only traces of 5-HT could be found in the erythrocytes. The concentrations of 5-HT in whole blood and in platelets before and at intervals after the burn are shown in Fig. 2 and 3. There is a marked decrease in the amounts of 5-HT in rabbit blood after burn injuries. There also is a prolonged diminution in the circulating number of blood platelets during the first hours after the burns (Fig. 2). The platelets, however, return to normal levels within 6 hours, but the decrease in the 5-HT content of blood remains for at least 12 hours. Platelet deficient plasma has, however, no detectable amounts of 5-HT. In 10^9 platelets however, an increase of the 5-HT content is found during the first hours after the burn injury.

Fig. 4 shows that 200 µg/kg of 5-HT given i. v. to three rabbits rapidly disappears from plasma even if a rather high dose of the amine is injected. More than 100 µg/kg of 5-HT creatinine sulfate must be injected before an increase in plasma content of 5-HT can be detected.

Clinical material. A determination is also made of the 5-HIAA content in 24 hours samples of urine and of 5-HT concentrations of the blisters in five patients with severe burns. The urinary determinations of 5-HIAA are given in Table I. The urinary excretion of 5-HIAA is considerably increased in these five patients with severe burns. The highest value measured is 35.2 mg 5-HIAA per 24 hours in one patient. The urinary excretion of 5-HIAA in this patient is shown

Table I. Excretion of 5-HIAA mg per 24 hours during the first 8 days after the burn accident

Case	E. H.	H. H.	S. N.	H. E.	I. Ö.
Age, years	53	19	45	5	58
Burnt area %	30—35	12	40	45	10
Deep 3rd degree burn..	30	4	10	20	6
1 day	5.3	16.2	15.2	10.4	9.2
2 »	16.1	12.0	15.8	14.2	8.3
3 »	18.9	35.0	14.3	24.8	5.1
4 »		17.8	33.9	7.2	3.8
5 »		12.1	6.9	13.9	9.8
6 »		11.2	18.6	11.8	9.0
7 »		19.8	19.9	7.0	7.1
8 »			11.0		50

in Fig. 5 as compared with the normal limits of 2—9 mg per 24 hours. (Mean value 4.8 ± 2.4 mg 5-HIAA per 24 hours in 30 healthy medical students of both sexes. Most of the blisters contain small amounts of 5-HT (about 0.1 μg per ml blister fluid). Not even traces of 5-HT could be detected in abdominal skin from 6 humans.

Human platelets are not saturated with 5-HT. *In vitro* incubation at 37° C with excess of 5-HT greatly enhances their content of the amine (Fig. 6).

Discussion

The present study indicates that an increase in the endogenous metabolism of 5-HT occurs after burn injuries. In rats there are increased amounts of 5-HT in abdominal skin after deep third degree burns. The function of 5-HT in the skin of the rat is not known, but it appears to be related to the reaction of the animal to stress (PARRATT and WEST 1957).

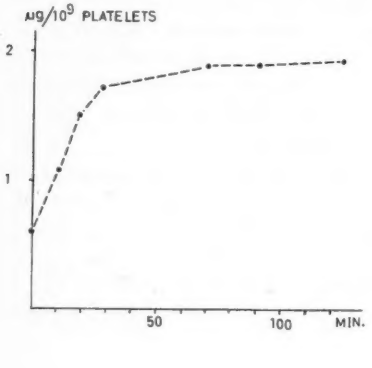

Fig. 6. 5-HT content of human platelets after incubation with excess of 5-HT.

In rabbits there is a marked diminution in the circulating number of platelets a few hours after the injury accompanied by a decrease in the whole blood concentrations of 5-HT. No release of 5-HT into platelet deficient plasma could be detected, probably due to the rapid metabolism of the amine. *In vivo* and *in vitro* studies have shown that platelets absorb 5-HT. This amine is liberated in the coagulation of blood, where the platelets are disrupted (ZUCKER and BORRELLI 1954). In most of the rabbits a slight increase of the 5-HT content in the circulating platelets is found and human platelets absorb the amine in vitro.

The burn injuries of the five patients are accompanied by an increase in the urinary excretion of 5-HIAA as an indication of an increased endogenous metabolism of 5-HT.

Almost all the 5-HT circulating in the blood is bound to the platelets (HUMPHREY and JAQUES 1954). A diminution of the circulating number of platelets thus produces a corresponding drop in the 5-HT content of the blood.

HEDINGER and LANGEMANN (1955) have shown that 5-HT in large doses injected into rats over a period of months increase the circulating number of platelets. The *in vivo* increase of 5-HT in blood platelets after injections of large doses of 5-HT persisted for days. No enzymes capable to form 5-HT have ever been found in the platelets. The platelets, however, contain a peroxidase, which *in vitro* reacts with a large number of substances of various kinds, and because their possible role in some hydroxylations, this group of enzymes might participate in the inactivation of 5-HT (JOHANSSON and PAUL 1959). Such facts suggest that the platelets are involved in mechanisms which inactivate liberated 5-HT in the blood.

Sensitized pigeons and guinea pigs are protected from the anaphylactic shock, if heparin is given 45 min before the anaphylactic dose (KEYS and STRAUSER 1926, WILLIAMS, RENE and VAN DER CARR 1927). Pretreatment of rabbits with high doses of heparin seems to prevent the release of 5-HT in burn injuries and anaphylactic reactions (JOHANSSON 1959).

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Further Observations on the Disruption of Rat Mesentery Mast Cells Caused by Compound 48/80, Antigen-Antibody Reaction, Lecithinase A and Decylamine

By

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Abstract

HÖGBERG, B. and B. UVNÄS. *Further observations on the disruption of rat mesentery mast cells caused by compound 48/80, antigen-antibody reaction, lecithinase A, and decylamine.* Acta physiol. scand. 1960. *48.* 133—145. — The influence of pH, ionic milieu, temperature and enzyme inhibitors on the disrupting action on rat mesentery mast cells of compound 48/80, antigen and decylamine has been investigated. A pH optimum around 7.4, the essentiality of Ca^{++} ions, the influence of temperature and the inhibitory action of various enzyme inhibitors, especially those blocking protein NH_2-groups and SH-groups, supported the view that compound 48/80 and antigen disrupt the mast cells by activating a lytic enzyme attached to the mast cell membrane. Decylamine, on the other hand, is a surface active agent that disrupts mast cells by a non-enzymatic mechanism.

Compound 48/80 is highly active in disrupting mast cells from the rat. In a previous paper we advanced the theory that the disruption is the result of an activation of a lytic enzymatic process hydrolyzing the lipoprotein phospholipids of the mast cell membrane (HÖGBERG and UVNÄS 1958). Later observations on mesentery mast cells from sensitized rats led to a similar proposition concerning the disruption caused by antigen-antibody reaction (HÖGBERG and UVNÄS 1958).

In the present paper we will add further evidence to support our enzyme theory. Since lecithinase A was found to be the only enzyme out of about

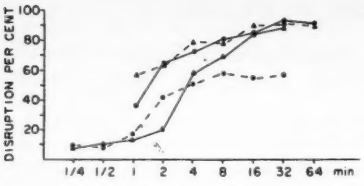

Fig. 1. Time course of mast cell disruption due to compound 48/80 0.5 μg/ml –▲– –▲– –▲–, lecithinase A 2 μg/ml –●—●—●– antigen (horse serum) 1 : 1,000 –●– –●– –●– and decylamine 20 μg/ml –▲—▲—▲–. Phosphate buffer.

35 so far tested that was able to disrupt mast cells in vitro, parallel studies have been performed on this "model enzyme". As a surface active agent able to disrupt mast cells in a non-enzymatic way decylamine was used.

Methods

Technique for Observations on Mast Cells. The technique was the same as that described by HÖGBERG and UVNÄS (1957) with the modification that the mesentery — when not otherwise stated — was incubated in a buffered solution (usually pH 7.4) containing $1.6 \cdot 10^{-1}$M NaCl, $3 \cdot 10^{-3}$M KCl, $9 \cdot 10^{-4}$M $CaCl_2$ (anhydrous) and 10 per cent Sörensen phosphate buffer ($3 \cdot 10^{-2}$M Na_2HPO_4, $2\,H_2O + 3.5 \cdot 10^{-2}$M KH_2PO_4). In experiments where inhibitors were used which, it was feared, could precipitate in the phosphate buffer, this buffer was exchanged for a 10 per cent barbitone buffer according to Michaelis.

Where inhibitors were investigated the mesentery was pre-incubated for 15 min together with the inhibitor before the disrupting substance was added. Some inhibitory substances were insoluble in water but were readily dissolved in ethyl alcohol, which could then be diluted with the incubation fluid. Care was always taken to keep the alcohol concentration below the inhibitory level (see Table II).

Sensitization of Rats. The rats were sensitized to horse serum by a subcutaneous injection of 0.5 ml horse serum mixed with 1.0 ml ($2 \cdot 10^{10}$ bacilli) pertussis vaccine. In the presence of this vaccine a marked sensitivity of the mast cells to horse serum developed within 8—10 days and persisted for at least 6 weeks (HÖGBERG and UVNÄS 1958).

Lecithinase A was obtained from bee venom and *compound 48/80* was synthetized as previously described by HÖGBERG and UVNÄS (1957).

Results

Time Course of Cell Disruption. In order to make possible at least semiquantitative observations the disruption time courses were studied for submaximal doses of the disruptive agents used. As seen from Fig. 1 the disruption does not occur instantaneously but develops successively. However, the incubation time used by us — 20 min — allows a maximal disruption to develop.

Influence of pH. When submaximal doses of compound 48/80 were used, the mast cell disruption curve showed a pH optimum around 7.5 (Fig. 2 a). The peak of maximal action was sharper in barbitone than in phosphate buffer.

The disruption due to antigen-antibody reaction and to lecithinase A

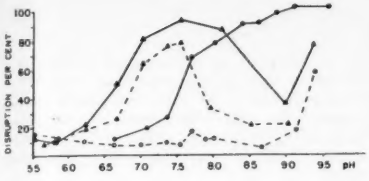

Fig. 2 a.

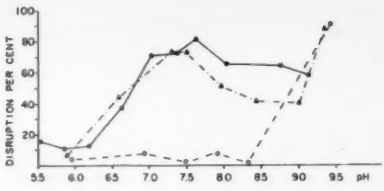

Fig. 2 b.

Fig. 2 a. Influence of pH on mast cell disruption due to compound 48/80 0.5 μg/ml –▲—▲—▲–, compound 48/80 0.1 μg/ml –▲– –▲– –▲– and decylamine 20 μg/ml –●—●—●–. Spontaneous disruption –○– –○– –○–. Barbitone buffer.
Fig. 2 b. Influence of pH on mast cell disruption due to antigen 1/125 ▲·–·▲·–·▲ and lecithinase A 0.5 μg/ml –●—●—●–. Spontaneous disruption –○– –○– –○–. Phosphate buffer.

(Fig. 2 b) showed less distinct pH optimum (the disruption curve tending to level off at a plateau on the alkaline side). The disruptive action of decylamine which was very weak on the acid side, steadily rose with increasing pH (Fig. 2 a). Above pH 9 observations on the action of disruptive agents were disturbed and finally made impossible due to an increasing tendency to spontaneous disruption.

Influence of Ionic Milieu. The mast cell disruption due to compound 48/80, antigen and lecithinase A was observed to require Ca^{++} ions. As seen from Table I, lack of Ca^{++} prevented the disruption. However, only minute amounts of Ca^{++} were necessary. In both phosphate and barbitone buffer a concentration of Ca^{++} about 10^{-3} M sufficed to restore the disruption ability of the three agents. On the other hand, decylamine did not require Ca^{++} for its action.

In accordance with the above findings a chelating agent such as ethylenediaminetetraacetate (EDTA) blocked the action of compound 48/80, antigen and lecithinase A but not that of decylamine (Table II).

Table I. Influence of Ca^{++} ions on mast cell disruption
Barbitone buffer

(Values Corrected for Spontaneous Disruption)

$CaCl_2$ mM	Disruption per cent			
	48/80 0.5 μg/ml	Lecithinase A 2 μg/ml	Antigen 1:1,000	Decylamine 20 μg/ml
0.01	2	5	14	59
0.1	28	30	26	59
0.5	59	69	84	57
1	65	87	80	61
5	62	84	85	62
10	64	83	86	54

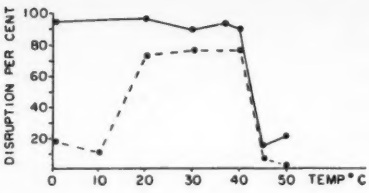

Fig. 3. Influence of temperature on mast cell disruption due to antigen. Mast cells incubated at different temperatures together with antigen –●– –●– –●–, mast cells pre-incubated at different temperatures before incubation at 37° C –●——●——●–. Barbitone buffer.

Mg^{++} ions were not required for the disruption of the mast cells, which proceeded seemingly undisturbed in the absence of these ions. On the contrary high concentration (10^{-2} M) reduced or blocked the disruption caused by compound 48/80, antigen and lecithinase A (Table III). High concentrations of Mg^{++} did not influence the action of decylamine (Table III).

Influence of Temperature. We have previously reported that the action of compound 48/80 is inhibited by prewarming the mast cells to a temperature between 40 and 50° C. This temperature inhibition was found to be irreversible; the mast cells being resistance to subsequent incubation with compound 48/80 at 37° C. However, the mast cells were observed still to be vulnerable to the action of lecithinase A and to surface active agents such as octylamine, digitonin and lysolecithin. The inhibition is explained as a denaturation of the assumed enzyme or its substrate.

In Fig. 3 two curves have been plotted to illustrate the influence of temperature on the disruption of mast cells due to antigen-antibody reaction. One curve shows the direct effect of the incubation temperature on the disruption rate, the other the disruption rate at 37° C after pre-incubation of the cells below and above 37° C. As seen the inhibitory effect of cooling the cells is reversible but that of heating is irreversible. Heating the cells to 50° C did not reduce the action of decylamine.

Influence of Enzyme Inhibitors. As previously reported, the action of compound 48/80 and lecithinase A was found to be blocked by enzyme inhibitors, among them reagents fairly specific for protein amino groups (HÖGBERG and UVNÄS 1957). The disruption caused by antigen was observed to be prevented by allicin (HÖBERG and UVNÄS 1958), a substance blocking SH-groups. These observations prompted us to a more extensive study of the effect of various metal ions and enzyme inhibitors on the mast cell disruption caused by 48/80, antigen, lecithinase A and decylamine. The results are presented in Table II.

Metal ions. Several bivalent metal ions were found to block strongly the mast cell disruption of compound 48/80, antigen and lecithinase A. Specially strong inhibitors were Pb^{++}, Cu^{++} and Zn^{++}, which caused a 50 per cent inhibition in concentrations between 10^{-7}—10^{-5} M. Moderately inhibitory were Cd^{++}, Co^{++}, Mn^{++} and Ni^{++}, which required concentrations between 10^{-5} and 10^{-3} M for 50 per cent inhibition. Slight or no inhibition was produced by Li^{+},

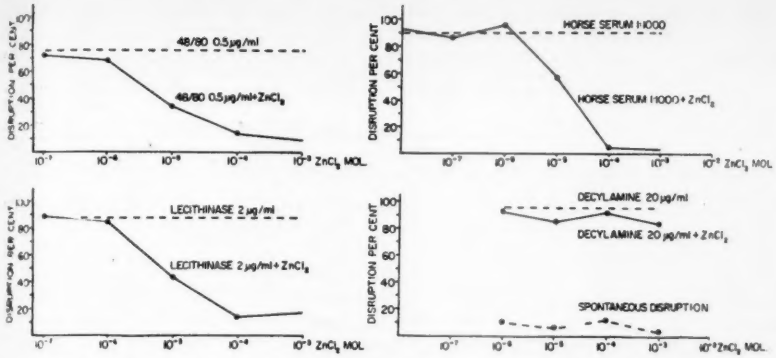

Fig. 4. Inhibitory action of Zn^{++} on mast cell disruption due to compound 48/80 0.5 µg/ml, lecithinase A 2 µg/ml, antigen 1/1,000 and decylamine 20 µg/ml. Barbitone buffer.

Mg^{++}, Hg^{++}, Pt^{++} and Al^{+++}. Fig. 4 illustrates the inhibitory action of Zn^{++}.

Metal ions did not in any case block the disrupting action of decylamine.

Amino Group Reagents. Dinitrofluorobenzene, formaldehyde, phenylisocyanate, acetic anhydride and ninhydrin in rather low concentrations produced a 50 per cent inhibition of the action of compound 48/80, antigen and lecithinase A.

The action of decylamine was not influenced.

SH Group Reagents. Iodoacetic acid, allicin, o-iodobenzoate, N-ethylmaleimide, and sodium arsenite all blocked the action of compound 48/80, antigen and lecithinase A. *p*-Chloromercuribenzoate on the other hand did not exert any appreciable inhibition (compare the negative effect of Hg^{++}).

The action of decylamine was not influenced.

Substances blocking oxidative energy generating processes. Sodium cyanide, dinitrophenol, L-thyroxine, 3,3,5-triiodo-L-thyronine and sodium azide blocked the action of compound 48/80, antigen and lecithinase A.

Various inhibitors. EDTA had a pronounced, phenol, thymol and p-thiocresol a certain blocking action on compound 48/80, antigen and lecithinase A.

The action of decylamine was not influenced.

Sodium arsenate, sodium fluoride, phlorizin, urethane, aminoguanidine and iproniazid lacked inhibitory action.

Ethyl alcohol had a diverging action. In phosphate buffer 1—4 per cent alcohol produced a 50 per cent inhibition of the action not only of compound 48/80 and lecithinase A but also of decylamine. Alcohol in barbitone buffer, on the other hand, did not exert any appreciable inhibition until the concentration exceeded 10 per cent.

Table II. Influence of Inhibitors on Mast Cell Disruption Caused by Compound 48/80, Antigen,

Inhibitor	Molar Concentrations of Inhibitors Exerting 50 per cent			
	48/80 0.5 μg/ml		Lecithinase A 2 μg/ml	
	P	Ba	P	Ba
Li Cl		No inhib. 10^{-2}		No inhib. 10^{-3}
$MgCl_2$	10^{-3}—10^{-2}		10^{-3}—10^{-2}	
$CdCl_2$		10^{-5}—10^{-4}		10^{-5}—10^{-4}
$MnCl_2$		10^{-5}—10^{-4}		10^{-5}—10^{-4}
$CoCl_2$		10^{-5}—10^{-4}		10^{-5}—10^{-4}
$NiCl_2$		10^{-4}—10^{-3}		10^{-4}—10^{-3}
$CuCl_2$		10^{-6}—10^{-5}		10^{-7}—10^{-6}
$ZnCl_2$		10^{-5}		10^{-5}
$HgCl_2$	10^{-4}—10^{-3}	10^{-3}	10^{-4}—10^{-3}	10^{-3}
$Pb(NO_3)_2$		10^{-6}—10^{-5}		10^{-6}—10^{-5}
Dinitrofluorobenzene	10^{-4}—10^{-3}	10^{-5}—10^{-4}	10^{-4}—10^{-3}	10^{-5}—10^{-4}
Formaldehyde	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
Phenylisocyanate		10^{-4}		10^{-4}
Acetic anhydride	10^{-4}—10^{-3}		10^{-3}	
Ninhydrin	10^{-5}—10^{-4}		10^{-5}—10^{-4}	
p-Chloromercuribenzoate		No inhib. 10^{-4}		No inhib. 10^{-4}
Allicin	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
Iodoacetate		10^{-4}—10^{-3}		10^{-4}
o-Iodosobenzoate	10^{-4}—10^{-3}	10^{-4}—10^{-3}		10^{-4}—10^{-3}
N-Ethylmaleimide	10^{-5}—10^{-4}		10^{-5}—10^{-4}	
Sodium arsenite	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
Sodium arsenate		No inhib. 10^{-2}		No inhib. 10^{-2}
EDTA	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
Dinitrophenol		10^{-5}—10^{-4}		10^{-5}
L-Thyroxine	10^{-5}—10^{-4}		10^{-5}—10^{-4}	
3,3,5-Triiodo-L-thyronine	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
Sodium azide	10^{-3}—10^{-2}		10^{-3}—10^{-2}	
Sodium cyanide		10^{-4}—10^{-3}		10^{-4}—10^{-3}
Phenol		10^{-3}—10^{-2}		10^{-3}—10^{-2}

P = phosphate buffer
Ba = barbitone buffer

Lecithinase A and Decylamine

Inhibition				
Antigen (Horse Serum 1/100–1/1000)		Decylamine 20 μg/ml		Remarks
?	Ba	P	Ba	
10^{-3}—10^{-2}		No inhib. 10^{-2}		
	10^{-5}—10^{-4}		No inhib. 10^{-2}	
	10^{-4}		No inhib. 10^{-3}	
	10^{-5}—10^{-4}		No inhib. 10^{-2}	
	10^{-5}—10^{-4}		No inhib. 10^{-4}	
	10^{-6}—10^{-5}		No inhib. 10^{-3}	Disrupt. in controls with $Cu^{++}>10^{-4}$
	10^{-5}		No inhib. 10^{-3}	
10^{-4}—10^{-3}		10^{-3}		Disrupt. in controls with $Hg^{++}>10^{-2}$. Mast cells appear shrunk in all Hg — conc. used
	10^{-6}—10^{-5}		No inhib. 10^{-3}	
10^{-4}—10^{-3}		No inhib. 10^{-3}		
10^{-4}—10^{-3}		No inhib. 10^{-2}		
	10^{-4}		No inhib. 10^{-3}	
10^{-4}				
10^{-5}—10^{-4}		No inhib. 10^{-2}		
	No inhib. 10^{-4}		No inhib. 10^{-4}	Disrupt. in controls with *p*-Chloromercuribenzoate 10^{-3}
10^{-4}—10^{-3}		No inhib. 2×10^{-3}		
10^{-4}—10^{-3}		No inhib. 10^{-2}	No inhib. 10^{-2}	Disrupt. in controls with iodoacetate 10^{-2}
10^{-4}—10^{-3}			No inhib. 10^{-3}	
		No inhib. 10^{-2}		
10^{-4}		No inhib. 10^{-2}		
No inhib. 10^{-2}		No inhib. 10^{-2}		
10^{-4}—10^{-3}		No inbib. 10^{-2}		Disrupt. in controls with EDTA 10^{-2}
	10^{-5}—10^{-4}		No inhib. 10^{-3}	
10^{-5}—10^{-4}		No inhib. 10^{-3}		
		No inhib. 10^{-3}		
10^{-3}—10^{-2}		No inhib. 10^{-2}		
	10^{-4}—10^{-3}		No inhib. 10^{-2}	
10^{-3}—10^{-2}		No inhib. 10^{-2}		

Table II. (Cont.) Influence of Inhibitors on Mast Cell Disruption Caused by Compound 48/

Inhibitor	Molar Concentrations of Inhibitors Exerting 50 per cent			
	48/80 0.5 μg/ml		Lecithinase A 2 μg/ml	
	P	Ba	P	Ba
Thymol	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
p-Thiocresol	10^{-4}—10^{-3}		10^{-4}—10^{-3}	
p-Cresol	10^{-3}—10^{-2}		10^{-3}—10^{-2}	
Sodium fluoride	No inhib. 10^{-4}		No inhib. 10^{-4}	
Phlorizin	No inhib. 10^{-3}		No inhib. 10^{-3}	
Iproniazid	10^{-3}—10^{-2}		10^{-3}—10^{-2}	
Aminoguanidine	No inhib. 10^{-2}			
Urethane	No inhib. 10^{-3}		No inhib. 10^{-3}	
Ethylalcohol	1—2%	No inbib. 10%	2—4%	No inhib. 10%
Hip seed compound	5—10 μg/ml		10—100 μg/ml	
Pk 11	1 μg/ml		5 μg/ml	

P = phosphate buffer, Ba = barbitone buffer

Hip seed polysaccharides (HÖGBERG *et al.* 1957) and a condensation product of salicylic acid (code number Pk 11[1]) blocked the action of compound 48/80 and lecithinase A. The polysaccharide had a slight but the synthetic polymer had no inhibitory action on antigen. Both were without effect on decylamine.

The inhibitory action of the lowest doses of Zn^{++}, allicin, dinitrofluorobenzene, phenol and hip seed polysaccharide that caused 100 per cent inhibition was tested against increasing doses of disrupting agents. As seen from Table III the inhibitory action of Zn^{++}, allicin and dinitrofluorobenzene remained comparatively unchanged in spite of a 100-fold increase in the compound 48/80 or the antigen concentration. An increase in the lecithinase A concentration, on the other hand, reversed the inhibition of allicin and dinitrofluorobenzene, while the blocking action of such inhibitors as phenol and hip seed polysaccharides was closely dependent on the concentration of the disrupting agents.

Washing the mesentery twice in inhibitor-free incubation fluid removed the inhibitory action of phenol and hip seed polysaccharides but did not reduce the inhibitory action of Zn^{++}, allicin and dinitrofluorobenzene.

[1] Preparation and properties of Pk 11 will be published elsewhere.

80, Antigen, Lecithinase A and Decylamine

Inhibition				
Antigen (Horse Serum 1/100—1/1,000)		Decylamine 20 μg/ml		Remarks
P	Ba	P	Ba	
10^{-4}—10^{-3}			No inhib. 10^{-3}	Disrupt. in controls with thymol 5×10^{-3}
10^{-3}		No inhib. 10^{-3} No inhib. 10^{-2}		
No inhib. 10^{-4}		No inhib. 10^{-4}		Disrupt. in controls with Sodium fluoride 10^{-3}
No inhib. 10^{-3}		No inhib. 10^{-3}		Disrupt. in controls with Phlorizin 10^{-2}
		No inhib. 10^{-3}		
No inbib. 10^{-3}		No inhib. 10^{-3}		Disrupt. in controls with Urethane 10^{-2}
2%		2—4%		
No inhib. 100 μg/ml		No inhib. 100 μg/ml		
No inhib. 100 μg/ml		No inhib. 100 μg/ml		

Influence of O_2-lack

Since the histamine release due to antigen-antibody reaction in sensitized guinea pig lung was found to depend on oxygen, and the disruption of mesentery mast cells to be blocked by oxidative inhibitors, mast cells were washed in N_2-saturated incubation fluid and then incubated in such buffer in N_2-atmosphere. The mast cell disruption due to compound 48/80, antigen, lecithinase A and decylamine proceeded apparently normally in such anaerobic milieu (Table IV).

Discussion

We have previously suggested the existence of a lytic enzyme attached to the mast cell membrane. This enzyme should be activated by cationic liberators such as 48/80 and protamine.

The present observations on the influence of pH, ionic milieu, temperature and several inhibitors agree with the theory.

So far the postulated lytic enzyme is not isolated from the mast cells and the nature of the enzyme is still to be clarified, but interesting analogies no doubt present themselves between the enzyme mechanism and the action of lecithinase

Table III. The Effect of Increasing Doses of Compound 48/80, Antigen and Lecithinase A on Inhibitory Action of Zn^{++}, Allicin, Dinitrofluorobenzene, Phenol and Hip Seed Polysaccharide

	48/80 μg/ml	Disruption per cent	Lecithinase A μg/ml	Disruption per cent	Antigen	Disruption per cent		48/80 μg/ml	Disruption per cent	Lecithinase A μg/ml	Disruption per cent
Control	0.5	83	2	89	1:1,000	94	Control	0.5	98	2	98
Zn^{++} 10^{-4} M	0.5	5			1:1,000	15	Hip seed 10 μg/ml	0.5	3	2	8
	1	8			1:500	10		1	10	4	32
	2	10	2	3	1:100	12		2	10	8	80
	4	8	4	4	1:50	18		4	97	16	100
	8	17	8	8	1:10	18		8	100	32	100
	16	9	16	11				16	100	64	100
	32	—	32	23				32	100	128	100
	64	5	64	15				64	100	256	100
Control	0.5	97	2	96	1:1,000	91	Control	0.5	99	2	89
Allicin 10^{-3} M	0.5	6	2	6	1:1,000	4	Dinitrofluorobenzene 10^{-3} M	0.5	11	2	9
	1	5	4	2	1:500	4		1	11	4	16
	2	10	8	5	1:100	6		2	24	8	75
	4	4	16	5	1:50	1		4	16	16	99
	8	11	32	10	1:10	7		8	15	32	100
	16	15	64	65				16	14	64	100
	32	7	128	90				32	31		
	64	2	256	100				64	27		
Control	0.5	95	2	98							
Phenol 10^{-2} M	0.5	12	2	10							
	1	23	4	60							
	2	70	8	84							
	4	80	16	100							
	8	92	32	100							
	16	100	64	100							
	32	100									
	64	98									

A on the mast cells. Lecithinase A is the only enzyme so far found by us to be able to disrupt mast cells in vitro. Striking similarities occur in the influence of various factors on the disruption of mast cells due to compound 48/80, antigen and lecithinase A. All three processes require Ca^{++} and Ca^{++} could not be replaced by other bivalent cations. They are inhibited by the same enzyme inhibitors. The importance of amino- and sulfhydryl groups for the

Table IV. Mast Cell Disruption in Oxygen and Nitrogen Atmosphere
(Values Corrected for Spontaneous Disruption)

Atmosphere	Disruption in per cent				
	48/80 0.5 μg/ml	Lecithinase A 2 μg/ml	Antigen 1:250	Decylamine 20 μg/ml	Control
N_2	68	63	85	83	17
O_2	74	67	85	89	9

full activity of both processes (HÖGBERG and UVNÄS 1957, 1958) has further been established. Also interesting to note is the strong inhibition of Cu^{++} and Zn^{++} ions on the processes since LONG and PENNY (1957) have observed that these ions exert an inhibition of lecithinase A.

It is impossible to establish definitely from our observations the mechanism of the inhibition of the various inhibitors. However, the fact that the inhibitory action of dinitrofluorobenzene, allicin and Zn^{++} seems to be rather independent of the dose of compound 48/80 or antigen but is overcome by increasing doses of lecithinase A is in agreement with the theory that these inhibitors react with the hypothetic mast cell enzyme and not with the substrate.

Preliminary experiments with lecithinase C, also a conceivable lytic enzyme, do not support the idea that this enzyme is identical with the mast cell enzyme. Lecithinase C is inhibited by fluoride, for example, but not by Zn^{++} ions. The action of these inhibitors — as shown above — on mast cell disruption is just the reverse. Specific antiprefringens serum (Behringwerke) which blocks the action of lecithinase C in vitro does not influence the disruption of mast cells due to compound 48/80, antigen and lecithinase A. This is in contrast to the inhibition produced by lecithinase A antiserum (HÖGBERG and UVNÄS 1957).

Clostridium prefringens toxin is reported by RILEY (1958), HABERMANN[1] (1958) and HÖGBERG and UVNÄS (1958) to disrupt mast cells in the rat mesentery. The disrupting ability has been ascribed to the lecithinase C content of the toxin. According to our experience lecithinase C has no disrupting action on mast cells. We believe the disrupting action of the toxin on the mast cells to be due to the presence of other factors in the toxin broth and not to lecithinase C. Our toxin broth contained for instance small amounts of lecithinase A (FREDHOLM, HÖGBERG and UVNÄS 1959, to be published).

Further evidence to support our "lytic enzyme" theory comes from observations on the occurrence and nature of SRS (Slow Reacting Substance) in the perfused cat's paw. When compound 48/80 was administered to the cat's paw,

[1] We thank Dr. E. HABERMANN for valuable discussions and exchange of preparations.

the output of histamine and SRS ran surprisingly parallel. Changes in temperature, the addition of enzyme inhibitors etc. produced parallel shifts in the occurrence of the two substances. The observations indicated that SRS is formed when mast cells are disrupted by the activation of the enzymatic mechanism (CHAKRAVARTY, HÖGBERG and UVNÄS 1959). Chemically SRS behaves as could be expected of a lipid of acidic character.

The liberation of histamine in anaphylactic reactions has been supposed to involve enzymatic activity (PARROT 1942, ROCHA E SILVA 1944, UNGAR 1947, MONGAR and SCHILD 1955). We have previously shown that an SH-reagent, allicin, is able to prevent the mast cell disruption caused by antigen in vitro (HÖGBERG and UVNÄS 1958). The present observations demonstrate a striking agreement between the influence of the temperature, effect of hydrogen ion concentration, influence of inorganic ions and enzyme inhibitors on the mast cell disruption due to compound 48/80 and antigen. CHAKRAVARTY and UVNÄS (1960) have found a good quantitative correlation between the appearance of histamine and SRS from sensitized guinea pig tissue on the addition of antigen in vitro under various experimental conditions. The SRS from sensitized guinea pig tissue and from cat's paw appeared to be biologically and chemically identical or closely related. We therefore believe that the enzymatic process initiated in anaphylactic reactions is essentially the same as that initiated by compound 48/80.

It has been observed by PARROT, MONGAR and SCHILD, ourselves and others that the histamine liberation due to anaphylactic reactions requires oxygen. Our efforts to show the same to be true for mesentery mast cells in vitro have been without success. Mast cell disruption due to compound 48/80, antigen or lecithinase A proceeded seemingly normally even in N_2-atmosphere and after washing the mesentery repeatedly in oxygen-free incubation fluid. We are not prepared to conclude from these observations that oxidative processes are unessential for the disruption of the mast cells caused by these agents. It might be that in our experiments the minute amounts of oxygen required have not been removed from the tissues or that energy yielding substances were present in sufficient amounts. The blocking action of dinitrophenol L-thyroxine, 3,3,5-triiodo-L-thyronine, sodium azide and sodium cyanide indicates that energy requiring phosphorylating processes are involved in the disruption mechanism started by compound 48/80, antigen and lecithinase A.

All observations on the disruptive action of decylamine indicate that this substance differs basically from the polymer amine compound 48/80. It seems to break the mast cell membrane in a nonenzymatic way.

It has to be stressed that our discussion is valid for the disruption mechanism of rat mesentery mast cells in situ, in other words, with the mast cells embedded in the connective tissue. The picture might change when studies are performed on isolated mast cells. Such studies are at present in progress (UVNÄS and THON 1959).

Rats differ from other animals such as guinea pigs in their susceptibility to sensitization. Rats are difficult to sensitize and adjuvance (*e. g.* pertussis vaccine) is required for a satisfactory sensitization to develop. Guinea pigs, on the other hand, are easily sensitized. Some investigators feel therefore that antigen-antibody reactions in these animal species differ profoundly from each other. We are not of this opinion, as far as the mechanism of the disruption of the mast cell goes.

Financial support from Stiftelsen Gustaf och Tyra Svenssons Minne to one of us (B. U.) is gratefully acknowledged.

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The Mechanism of Histamine Release in Anaphylactic Reaction in Guinea Pig and Rat

By

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Abstract

CHAKRAVARTY, N. *The mechanism of histamine release in anaphylactic reaction in guinea pig and rat.* Acta physiol. scand. 1960. *48.* 146—166. — The mechanism of histamine release was studied in anaphylactic reaction *in vitro* in tissues from guinea pig and rat; it was compared, in rat, with histamine release induced by compound 48/80. The observations suggest that histamine release in both species is mediated by the activation of an enzyme system probably with essential sulphydryl and amino groups. Histamine release in anaphylaxis and that induced by compound 48/80 in rat appear to be mediated by the same mechanism. Some differences were observed in anaphylactic histamine release in the two species: anoxia inhibited histamine release in guinea-pig tissue but not in rat tissue; the same was true of two triphenylmethane dyes. Despite these differences, anaphylactic histamine release in the two species seems to follow, on the whole, a common pathway.

In recent years a number of theories have been advanced pointing to an enzymatic mechanism for histamine release. The nature of the enzyme system, however, is still not clearly defined, although it was suggested earlier that the activation of proteolytic enzymes results in splitting of histamine from a peptide linkage (ROCHA E SILVA 1938, UNGAR 1947). MONGAR and SCHILD (1957) found that temperatures above 45° C, which activate proteolysis, block the anaphylactic mechanism. Despite this conflicting view they also

pointed to an enzymatic mechanism for histamine release. HÖGBERG and UVNÄS (1957) propounded the theory that compound 48/80 caused degranulation of rat mast cells through activation of a lytic enzyme; and the observations of CHAKRAVARTY, HÖGBERG and UVNÄS (1959) that compound 48/80 releases histamine and a lipid-soluble acid ("SRS") in the cat by an enzymatic process were consistent with such a view. It was also observed in rat that mast cell degranulation caused by antigen and that caused by compound 48/80 were equally blocked by certain enzyme inhibitors (HÖGBERG and UVNÄS 1958, 1960).

In the present experiments the mechanism of anaphylactic histamine release in guinea pig and rat tissue was investigated, particularly to determine if an enzymatic reaction is involved. It was also sought to ascertain if the process of histamine release in the rat is similar in anaphylaxis to that induced by compound 48/80. By altering the external milieu of the tissue the conditions for *in vitro* histamine release were investigated and the effects of a few enzyme inhibitors were studied. The results suggest activation of an enzyme system with essential sulphydryl and amino groups, initiating histamine release in anaphylactic reaction. Histamine release, produced in rat by anaphylaxis and by compound 48/80, appears to be mediated by the same or very similar processes.

Materials and Methods

Guinea pigs (male or female, 250 to 350 g) were sensitized by two injections, subcutaneously and then intraperitoneally, of 0.1 g crystalline egg albumin with an interval of 3 to 7 days. Anaphylactic reaction was produced in the isolated tissues — cut into small pieces — *in vitro* 4 to 14 weeks after the first sensitizing dose. Rats (male or female, 150 to 200 g) were sensitized to horse serum by injections of horse serum, 0.5 ml subcutaneously and 0.5 ml intraperitoneally, and *H. pertussis* vaccine, 1 ml intraperitoneally (2×10^{10} bacilli per ml) on the same day. Isolated lung pieces were used for *in vitro* anaphylactic reaction 2 to 6 weeks after the sensitizing dose. Compound 48/80 was used either in the same sensitized rats or in rats of like weight.

The lungs were used for all experiments except those in which other tissues were specifically investigated for histamine release. Anaphylactic reaction was produced *in vitro* by a modification of the method of UNGAR and PARROT (1936). Guinea pigs were stunned by a light blow on the head and bled from the axillary artery. Rats were lightly anaesthetized with ether and similarly bled. In this way it was possible to avoid aspiration of blood into the respiratory tract. The lungs from 2 to 4 animals were removed and adjacent pieces of each lobe were divided into 6 to 10 equal parts so as to ensure a more or less uniform distribution of tissues in the samples. They were weighed, cut into smaller pieces (1—2 mg) and washed with Tyrode solution. Lastly they were suspended in a water bath at 37° C in Tyrode solution with antigen or compound 48/80 and mechanically shaken. Guinea pig tissue was incubated in oxygenated Tyrode solution (10 ml/g tissue) with 1 mg/ml egg albumin. Rat tissue was similarly incubated with 1 : 50 to 1 : 200 horse serum or 20 μg/ml compound 48/80. A Warburg apparatus with 25 ml Erlenmeyer flasks for incubation of the tissue was found convenient for the experiments. The samples were withdrawn from the bath after in-

cubation with the antigen for 10 min, filtered through cotton wool and the filtrates immediately cooled to 0° C. They were stored at — 20° C until used for testing histamine. This general plan had to be modified slightly in some experiments as described in the relevant sections. The spontaneous release of histamine was usually low and, when detectable, was deducted to obtain the release value in anaphylaxis or the release induced by compound 48/80. — The values are all corrected for spontaneous release with the exception of those relating to anoxia (Fig. 8), where the spontaneous release is also recorded. In duplicate samples, the amounts of histamine released in anaphylactic reaction and by compound 48/80 showed only small differences (see CHAKRAVARTY 1960) thus permitting the evaluation of the effect of inhibitors or other experimental procedures.

When an inhibitor was used the tissue was incubated with the inhibitor for 15 to 20 minutes, before exposure to the antigen. In some experiments the tissues were washed two or three times in a relatively large volume of Tyrode solution following incubation with inhibitor, and resuspended in Tyrode solution before the releaser was added. The inhibitors, 2:4-dinitrofluorobenzene and phenylisocyanate were dissolved in a small volume of ethyl alcohol before they were obtained in aqueous solution; the amount of alcohol used with the inhibitor had no effect on histamine release.

Residual histamine in the tissue was estimated by boiling the pieces in 10 ml/g water and 2 ml/g N HCl for three minutes. The extract was filtered, neutralized and brought to a suitable volume for testing.

Histamine was assayed biologically on atropinized (1.5×10^{-6} M atropine sulphate) guinea-pig ileum. All histamine values are expressed as histamine base. The presence of antigen and compound 48/80, in the concentrations used, did not interfere with the estimation of histamine. When inhibitors, reactivators, etc. were used, they were added in equal amount to the standard. When ethyl alcohol was employed as an inhibitor the samples were freeze-dried and redissolved in water before being tested on guinea-pig gut. In the case of another inhibitor, allicin, the samples were heated in a boiling water bath for 10 minutes to avoid interference with the estimation of histamine.

Hip-seed polysaccharide was prepared from air-dried ripe hips by the method described by HÖGBERG, *et al.* (1957).

Allicin was extracted from garlic (*Allium sativum*) by the method of CAVALLITO and BAILEY (1944).

Pk 11 and Pk 312 are the code names for polymers of salicylic acid and o-aminobenzoic acid respectively. These substances, as well as polyphloretin phosphate and compound 48/80 (condensation product of p-methoxyphenethylmethylamine with formaldehyde), were supplied by AB Leo, Hälsingborg, Sweden.

Results

Time Course. The time course was investigated by incubating guinea pig lung pieces with antigen, and withdrawing small amounts of incubation fluid at intervals till the end of the experiment. Histamine appeared in the incubation fluid within a few seconds, and rapidly rose to a peak between 8 and 16 min. The early phase of histamine release is shown in Fig. 1. To judge by the curve, histamine release started after a latency of a few seconds (about 15 sec by extrapolation of the curve) and proceeded initially at its maximum rate; then progressively slowed down until it was nearly complete at about

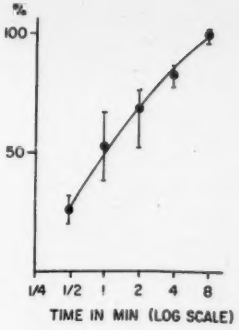


Fig. 1. Time course of anaphylactic histamine release from guinea pig lung pieces. The release is expressed as per cent of the maximum amount found in the incubation fluid at any time up to 16 minutes or more. The mean and range are shown from 2 experiments at 1/2 min and from 5 experiments at other periods.

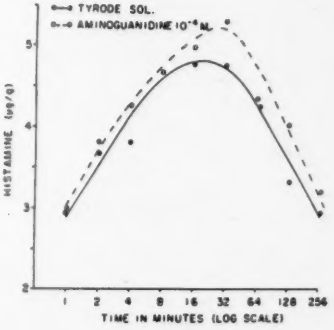


Fig. 2. Time course of anaphylactic histamine release from guinea pig lung pieces incubated with antigen for 256 min and the effect of aminoguanidine on it. Continuous line: tissue incubated in Tyrode solution. Broken line: tissue incubated with 10^{-4} M aminoguanidine in Tyrode solution.

8 min. When incubation was continued longer than 16 min, not only was there no further release of histamine but its value actually diminished. This is apparent from the experiment shown in Fig. 2 in which incubation was continued over 4 hours; 38 per cent of the released histamine was inactivated in 256 min. In order to study the possibility of enzymatic degradation, 10^{-4} M aminoguanidine was added to Tyrode solution, in one experiment, to inhibit histaminase; and in another 10^{-4} M iproniazid (marsilid), an inhibitor of monoamine oxidase, was added. It will be seen from Fig. 2 and 3 that neither had a marked effect upon the degradation of histamine on prolonged incubation of the tissue with antigen. The question of acetylation was also investigated. In one experiment where 39 % of the released histamine was inactivated in 128 min, the final incubation fluid was hydrolyzed by boiling for 1 1/2 hours with concentrated hydrochloric acid according to the method of ANREP *et al.* (1944). No increase in free histamine resulted from this procedure, as would have been expected if acetylation played an appreciable role. The possibility of degradation by bacterial action was investigated by adding phenol, 1 : 500 (2.1×10^{-2} M), 9 minutes after addition

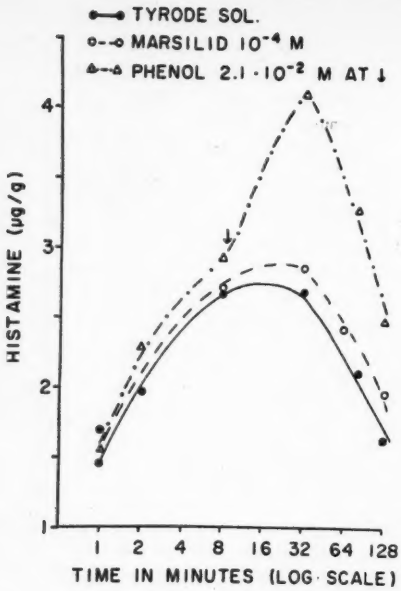


Fig. 3. Histamine release from guinea pig lung pieces incubated with antigen in Tyrode solution: effect of iproniazid (marsilid) and phenol on the decrease of histamine value in the incubation fluid on prolonged incubation. Phenol was added at the arrow.

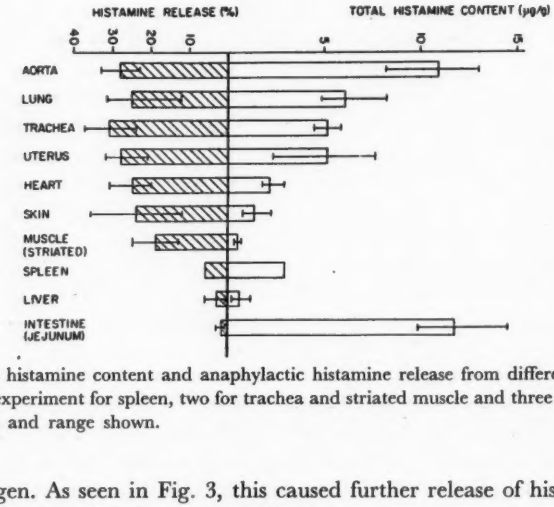


Fig. 4. Total histamine content and anaphylactic histamine release from different guinea pig tissues. One experiment for spleen, two for trachea and striated muscle and three for the others. Mean values and range shown.

of the antigen. As seen in Fig. 3, this caused further release of histamine and its subsequent inactivation was unaffected.

Histamine Release from Different Tissues. In spite of a wide range of total histamine contents in different tissues of the guinea pig, the percentage of

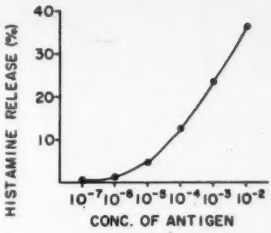


Fig. 5. Effect of concentration of antigen (w/v) on anaphylactic histamine release from guinea pig lung. Per cent of total histamine released is shown.

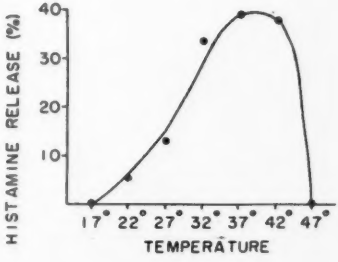


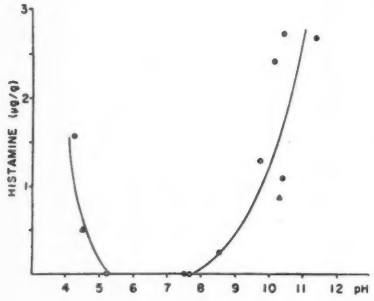
Fig. 6. Effect of temperature (°C) on anaphylactic histamine release from guinea pig lung. The figures show per cent of total histamine released.

histamine released from aorta, lung, trachea, uterus, heart and skin (abdominal) was about the same, viz. one-third to one-fourth of the total histamine (see Fig. 4). The histamine content in the liver was low and little was released. In striated muscle, on the other hand, the histamine content was equally low but about one-fifth was released. The histamine release from spleen was found in one experiment to be rather poor. The intestine (jejunum) differed greatly from all other tissues investigated in that its histamine content was very high while its release was low (average, 1.5 % in three experiments).

Concentration of Antigen. The effect of concentration of antigen on histamine release from lung pieces of sensitized guinea pig are shown in Fig. 5. The release of histamine was very low with the lowest concentrations of antigen (10^{-6} and 10^{-7}) but it then rose almost linearly with log dose of the antigen. The highest concentration of antigen released 2.1 μg/g histamine, amounting to 36 per cent of the total.

Temperature. When lung pieces from sensitized guinea pigs were incubated with antigen at different temperatures, histamine was not released at 17° C, but thereafter was released gradually to a maximum at 37° C, and at 47° C ceased to occur (see Fig. 6). On incubating the tissues at different temperatures for 10 min and bringing them to 37° C for anaphylactic reaction, it was observed that at lower temperatures no irreversible change occurred in the tissue to interfere with the reaction; but heating to 45° C or more did produce an irreversible change, and subsequent anaphylactic histamine release at 37° C

Fig. 7. (a) Influence of pH on anaphylactic histamine release from guinea pig lung: 3 experiments; values expressed as per cent of maximum release in each experiment.



(b) Influence of pH on spontaneous histamine release from guinea pig lung: 4 experiments.

was only 5 % of the value of the control in which preliminary heating had been omitted.

Effect of pH. The effect of pH on histamine release in anaphylactic reaction is shown in Fig. 7. Lung pieces from sensitized guinea pigs were incubated in 0.85 % sodium chloride solution containing 1.8×10^{-3} M calcium chloride, and buffered with one-tenth its volume of isotonic veronal buffer. A few drops of dilute (0.1 to 1 N) HCl or NaOH were added to the buffer solution, when necessary, to obtain the desired pH range. The tissues were washed in the solutions with different pH for 25 min and then incubated again with antigen in fresh solutions with the respective pH. The buffering action of the tissue caused some change in the pH of the solution following incubation. The final pH of the solution after incubation with the antigen (determined electrometrically) is recorded in the figure. Histamine, as shown in the figure, was spontaneously released at pH above 8 or below 5. Therefore the pH curve for anaphylactic histamine release could be completed on the alkaline side only after deduction of the amounts spontaneously released in control samples. It will be seen that anaphylactic histamine release is restricted between pH 5.6 and 9.5 with the optimal value at pH 7—8.5.

Effect of Oxygen Lack. Anaphylactic reaction is believed to be dependent on oxidative metabolism (Parrot 1942, Mongar and Schild 1957). The effects

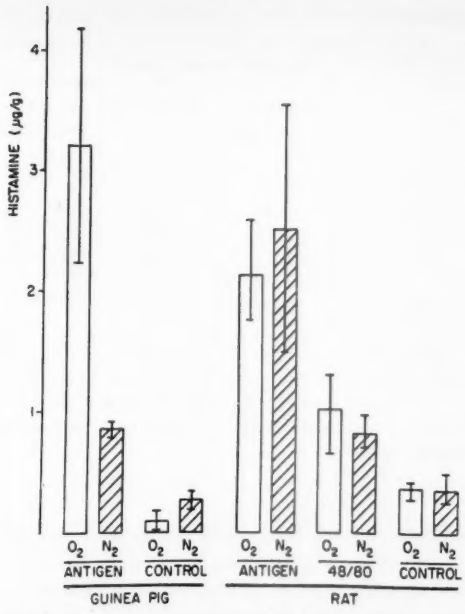


Fig. 8. Effect of oxygen lack on histamine release from guinea pig and rat lungs. Anoxia was produced by bubbling nitrogen through the solution. Control = spontaneous histamine release. Mean and range are shown from two experiments in guinea pig and for three experiments in rat.

of oxygen lack on anaphylactic histamine release in guinea pig and rat in the present investigation is shown in Fig. 8. When antigen was added to guinea-pig lung pieces suspended in oxygen-free (nitrogen-saturated) Tyrode solution, 71 to 86 % of histamine release was inhibited. On the other hand in rat lung pieces oxygen lack caused no reduction of anaphylactic histamine release. Similarly, anoxia had no appreciable effect on compound-48/80-induced histamine release from rat lung.

Lack of Calcium. HUMPHREY and JAQUES (1955) reported that free calcium ions were necessary for the release of histamine and 5-hydroxytryptamine from rabbit platelets by antigen-antibody reaction. To study the effect of calcium lack on histamine release in guinea pig and rat, lung pieces were suspended in 0.85 % NaCl buffered with veronal or phosphate buffer to give a pH of about 8. The histamine release (for anaphylactic reaction in guinea pig) in this solution containing 1.8×10^{-3} M calcium chloride was only slightly lower than that in Tyrode solution. It will be seen from Table I that EDTA, in a concentration of 2×10^{-4} M to 3.4×10^{-4} M, almost completely inhibited histamine release in anaphylaxis in both guinea pig and rat; histam-

Table I. Role of calcium on histamine release in guinea pig and rat tissue

Expt. No.		Releaser	Histamine release (μg/g)		
			(A) In presence of calcium	(B) EDTA[1]	(C) Same as B but Ca added later
Guinea pig	1	Antigen	1.8	0.24	1.3 ($CaCl_2$ 10^{-2}M)
	2	»	0.79	0	1.2 »
	3	»	1.7	0	1.2 ($CaCl_2$ 3.6×10^{-3}M)
Rat	4	Antigen	0.58	0	—
	5	»	1.2	0	1.3 ($CaCl_2$ 3.6×10^{-3}M)
	6	Compd. 48/80	1.1	0.05	0.58 ($CaCl_2$ 10^{-2}M)

Phosphate buffer was used for No. 2 and veronal buffer for all the others.

[1] EDTA (Ethylenediaminetetraacetic acid) was used in a concentration of 2×10^{-4}M to 3.4×10^{-4}M in calcium-free solution.

ine release induced in rat by compound 48/80 was similarly inhibited. The role of calcium is shown by the reappearance of 55 to over 100 % of the release without EDTA when 3.6×10^{-3} M to 10^{-2} M $CaCl_2$ was added after 10 min incubation with EDTA and antigen, and incubation continued for another 10 min. In guinea-pig tissue the subsequent addition of 2 to 10 mM magnesium chloride was similarly investigated and found ineffective in restoring the histamine release inhibited by EDTA.

Inhibition of Histamine Release by Enzyme Inhibitors. Polymeric substances like hip-seed polysaccharide (HSP), Pk 11 and Pk 312 (see p. 148) have been shown to inhibit the release of histamine by compound 48/80 (HÖGBERG *et al.* 1957, CHAKRAVARTY, HÖGBERG and UVNÄS 1959, HÖGBERG and UVNÄS 1960). They probably act by enzymatic inhibition, presumably of a nonspecific nature. The effects of these compounds on anaphylactic histamine release from guinea-pig lung were studied. Fig. 9 shows that both HSP and Pk 11 are effective inhibitors of histamine release in anaphylaxis in a concentration of 10^{-3} w/v; in this concentration Pk 312 has an even more striking effect, as shown in Fig. 10. Another polymeric substance — polyphloretin phosphate — prepared from phloretin, the aglucone of phlorrhizin, has a molecular weight of about 15,000 and is a powerful inhibitor of certain enzymes (DICZFALUSY *et al.* 1953). — Like the other polymeric substances, it inhibited anaphylactic histamine release from guinea-pig lung pieces in a concentration of 10^{-3} but not 10^{-4} (w/v). In sensitized rats the results were similar, but histamine release from rat lung in response to compound 48/80 was inhibited even in a concentration of 2×10^{-4} polyphloretin phosphate (w/v). These results are presented in Table II, which also shows the effect of other enzyme inhibitors. Iodoacetate

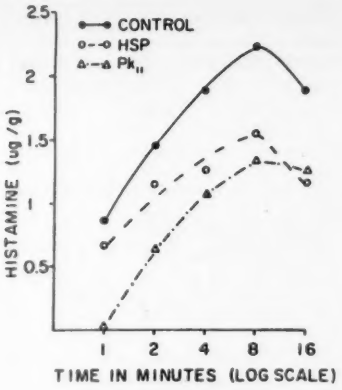


Fig. 9. Inhibition of anaphylactic histamine release from guinea pig lung by hip seed polysaccharide (HSP) and polysalicylic acid (Pk 11), 10^{-3} w/v. Control = lung tissue incubated with antigen only.

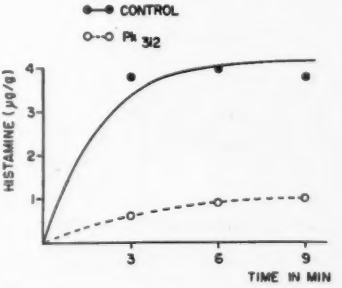


Fig. 10. Inhibition of anaphylactic histamine release from guinea pig lung by Pk 312 (formaldehyde condensation product of o-aminobenzoic acid), 10^{-3} w/v. Control = lung tissue incubated with antigen only.

and allicin, which combine with sulphydryl groups of enzymes, were very effective inhibitors of histamine release in a concentration of 10^{-3} M or even lower. With iodoacetate (10^{-3} M) the average inhibition of histamine release from guinea-pig lung in five experiments was 86 %. Allicin in a concentration of 7×10^{-4} M to 10^{-3} M in four experiments produced 74 to 88 % inhibition of histamine release from guinea-pig lung. Iodoacetate was used in rats in a concentration of 10^{-3} M and was found to be equally effective for blocking histamine release both in anaphylaxis and in that induced by compound 48/80.

The specific blocking of sulphydryl groups by iodoacetate and allicin is further demonstrated by the experiment with glutathione and cysteine as shown in Table III. The tissues (sensitized guinea pig lung) were incubated with the reactivator for 15 min before addition of the inhibitor; antigen was added after another 15 min. It will be seen from the table that, both with iodoacetate and allicin, there was an increase in the histamine release in incubation fluids wherein a reactivator was used with the inhibitor. The re-

Table II. Inhibition of histamine release by enzyme inhibitors in guinea pig and rat tissue

	Inhibitor	Concentration	Releaser	Histamine release		
				Without inhibitor (μg/g)	With inhibitor (μg/g)	With inhibitor as % of control (without inhibitor)
Guinea pig	Polyphloretin phosphate	2×10^{-3}w/v	Antigen	3.1	0.60	19
		10^{-3}w/v	»	3.7	1.5	41
		10^{-4}w/v	»	3.7	2.8	76
		10^{-5}w/v	»	3.7	4.2	114
	2:4-dinitro-fluorobenzene	§ 10^{-3}M	»	1.9	0	0
		10^{-4}M	»	3.2	1.0	31
		10^{-4}M	»	3.1	0.56	18
		10^{-5}M	»	3.2	3.0	94
	Phenylisocya-nate	10^{-2}M	»	2.9	1.4	48
		10^{-3}M	»	7.6	4.2	55
	Acetic anhydride	§ 10^{-2}M	»	1.9	0	0
		§ 10^{-2}M	»	3.5	0.10	3
		10^{-3}M	»	7.6	6.2	82
	Iodoacetate	10^{-2}M	»	3.7	0.15	4
		10^{-3}M	»	3.7	0.83	22
		10^{-3}M	»	1.3	0.16	12
		10^{-3}M	»	2.1	0.35	17
		10^{-3}M	»	6.2	0.89	14
		10^{-3}M	»	7.6	0.24	3
		5×10^{-4}M	»	2.1	0.45	21
		10^{-4}M	»	3.7	2.9	78
	Allicin	7×10^{-3}M	»	4.2	0.70	17
		10^{-3}M	»	1.2	0.14	12
		10^{-3}M	»	1.2	0.15	13
		10^{-3}M	»	6.6	0.91	14
		7×10^{-4}M	»	4.2	1.1	26
		10^{-4}M	»	6.6	5.8	88
		7×10^{-5}M	»	4.2	4.8	114
	Phlorrhizin	10^{-2}M	»	3.1	2.7	87
		10^{-3}M	»	3.7	3.6	97
		10^{-4}M	»	3.7	4.1	111
	Urethane	10^{-2}M	»	3.7	2.8	76
		10^{-3}M	»	3.7	3.9	105
		10^{-4}M	»	3.7	4.0	108
	Malonic acid	10^{-2}M	»	3.1	2.7	87

	Inhibitor	Concentration	Releaser	Histamine release		
				Without inhibitor (μg/g)	With inhibitor (μg/g)	With inhibitor as % of control (without inhibitor)
Rat	Polyphloretin phosphate	2×10^{-3}w/v	Antigen	1.2	0.51	43
		2×10^{-4}w/v	»	1.5	0.95	63
		2×10^{-3}w/v	Compd. 48/80	2.4	0.34	14
		2×10^{-4}w/v	»	2.4	0	0
	2:4-dinitro-fluorobenzene	§ 10^{-3}M	Antigen	1.2	0.15	13
		§ 10^{-3}M	Compd. 48/80	0.69	0.25	36
	Phenylisocyanate	10^{-2}M	Antigen	1.2	0.59	49
		10^{-2}M	Compd. 48/80	0.69	0.18	26
	Acetic anhydride	§ 10^{-2}M	Antigen	1.2	0.13	11
		§ 10^{-2}M	Compd. 48/80	0.69	0.12	17
	Iodoacetate	10^{-3}M	Antigen	1.7	0.06	4
		10^{-3}M	Compd. 48/80	2.4	0.44	18

§ Tissue washed after incubation with inhibitor.

Table III. Reversal by reduced glutathione and cysteine of the inhibitory effect of iodoacetate and allicin on anaphylactic histamine release in guinea pig lung

Inhibitor		Reactivator		Histamine release	
				With inhibitor as % of control (without inhibitor)	With inhibitor + reactivator as % of control (without inhibitor)
Iodoacetate	10^{-3}M	Glutathione	8×10^{-3}M	13	36
»	10^{-3}M	»	4×10^{-3}M	16	22
»	5×10^{-4}M	»	4×10^{-3}M	21	41
»	10^{-3}M	Cysteine	4×10^{-3}M	16	29
»	5×10^{-4}M	»	4×10^{-3}M	21	41
Allicin	10^{-3}M	Glutathione	8×10^{-3}M	13	77
»	10^{-3}M	»	8×10^{-3}M	12	69
»	10^{-3}M	»	1.6×10^{-3}M	12	49
»	10^{-3}M	Cysteine	8×10^{-3}M	12	76
»	10^{-2}M	»	$1.6\times10^{3-}$M	12	37

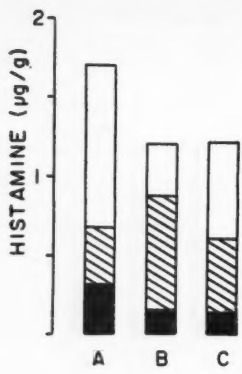


Fig. 11. Inhibition of anaphylactic histamine release from guinea pig lung by iodoacetate and allicin and its partial reversal by reduced glutathione. The total height indicates histamine release without inhibitor and the solid block shows release with inhibitor; the solid + shaded blocks represent histamine release in presence of both inhibitor and glutathione. A: Mean of two expts. Inhibitor: iodoacetate 5×10^{-4} M and 10^{-3} M; glutathione, 8 times higher molar conc. B: Mean of two expts. Inhibitor: allicin 10^{-3} M; glutathione 8×10^{-3} M. C: One expt. Inhibitor: allicin 10^{-3} M; glutathione 1.6×10^{-3} M.

versal of iodoacetate- and allicin- inhibition of histamine release by glutathione is also illustrated in Fig. 11.

Some enzyme inhibitors, which are supposed to react with amino groups, blocked histamine release. As shown in Table II, 2:4-dinitrofluorobenzene (DNFB) inhibited histamine release in a concentration of 10^{-3} M to 10^{-4} M, and the effect persisted even when the tissue was washed with Tyrode solution before the addition of antigen. It was effective against anaphylactic histamine release in guinea pig and rat, and against compound-48/80-induced histamine release in rat. DNFB combines with the terminal amino group of proteins to form dinitrophenyl-(DNP-)protein, as shown by SANGER (1945), but other reactions are also possible. Phenylisocyanate and acetic anhydride readily combine with the amino groups of proteins to form phenylureido and acetyl derivatives respectively. It will be seen in Table II that when lung pieces from sensitized guinea pigs were incubated with 10^{-2} M acetic anhydride, washed and resuspended in Tyrode solution with antigen, histamine release was almost completely inhibited. Phenylisocyanate in a concentration of 10^{-2} M to 10^{-3} M caused about 50 per cent inhibition of histamine release from guinea-pig lung. Experiments with both these reagents on rat tissue showed very similar results.

Since histamine release in the guinea pig depends on oxidative metabolism, malonic acid, which blocks the utilization of succinate in the tricarboxylic acid cycle, was tested and found to be ineffective in inhibiting histamine release; phlorrhizin, probably an inhibitor of phosphorylase and urethane, which causes inhibition of dehydrogenases (HADDOW and SEXTON 1946), were also shown to have no effect on histamine release from sensitized guinea-pig tissue.

Inhibition of Histamine Release by Ethyl Alcohol. Some of the reagents used in the present experiments had to be dissolved in a small volume of ethyl alcohol before they could be obtained in aqueous solution. The effect of alcohol itself

Table IV. Inhibition of histamine release by ethyl alcohol in guinea pig and rat tissue

	Releaser	Conc. of inhibitor (v/v)	Histamine release		
			Without inhibitor (μg/g)	With inhibitor (μg/g)	With inhibitor as % of control (without inhibitor)
Guinea pig	Antigen	2×10^{-2}	2.4	0.21	9
		§ 2×20^{-2}	2.4	2.6	108
		10^{-2}	6.2	2.9	47
		10^{-2}	2.4	0.94	39
		10^{-2}	2.9	0.95	33
		10^{-2}	3.1	1.1	35
		10^{-3}	2.4	2.7	113
Rat	Antigen	2×10^{-2}	0.74	0.05	7
		10^{-2}	1.7	0.59	35
	Compd. 48/80	2×10^{-2}	0.89	0.17	19
		§ 10^{-2}	2.4	1.8	75

§ Tissue washed after incubation with inhibitor.

on histamine release had therefore to be investigated. Although the amount of alcohol used with other reagents (see p. 148) had no effect, it inhibited histamine release in a concentration of 1 : 100 or more. Ethyl alcohol 1 : 50 caused 91 to 93 % inhibition of anaphylactic histamine release in rat and guinea-pig lung pieces; a concentration of 1 : 100 produced 53 to 67 % inhibition, and 1 : 1000 alcohol was ineffective, as shown in Table IV. The inhibitory effect of ethyl alcohol disappeared when the tissue was washed before the addition of antigen. The effect of alcohol on compound-48/80-induced histamine release was investigated in two experiments and found to be similar.

Inhibition of Histamine Release by Organic Dyes. NIKOLAEFF and GOLDBERG's (1930) observation that anaphylactic reaction in the guinea pig could be inhibited by pretreatment with congo red was subsequently confirmed by GORDON and ROBSON (1932) and, more recently, by DENZUMI (1958). Some other azo dyes (methyl orange, trypan blue, tropaeolin 0) have been shown to inhibit disruption of rat mesentery mast cells by compound 48/80 (FREDHOLM 1959). In the present investigation a few organic dyes were investigated for possible effects on histamine release. The following dyes of three different types were tested to ascertain if specific groups might affect the process of inhibition:

A. Azo dyes: congo red, trypan blue, methyl orange
B. Anthraquinone dyes: sodium alizarin sulphonate
C. Triphenylmethane dyes: rhodamine B, methyl green

Table V. Inhibition of histamine release by organic dyes in guinea pig and rat tissue

	Inhibitor	Concentration	Releaser	Histamine release		
				Without inhibitor (μg/g)	With inhibitor (μg/g)	With inhibitor as % of control (without inhibitor)
Guinea pig	Congo red	10^{-3}M	Antigen	3.5	1.7	49
		10^{-3}M	»	2.9	1.6	55
		§ 10^{-3}M	»	2.5	1.6	64
		§ 10^{-3}M	»	2.4	2.3	96
	Trypan blue	10^{-3}M	»	2.9	1.1	38
		§ 10^{-3}M	»	2.4	1.5	63
	Methyl orange	2×10^{-3}M	»	3.5	0.80	23
		2×10^{-3}M		2.9	1.1	38
		§ 2×10^{-3}M	»	2.5	1.7	68
		§ 2×10^{-3}M	»	2.4	1.7	71
	Sodium alizarin sulphonate	2×10^{-3}M	»	2.9	2.1	72
		§ 2×10^{-3}M	»	2.4	1.9	79
		10^{-3}M	»	3.5	2.2	63
		§ 10^{-3}M	»	2.4	1.7	71
	Rhodamine B	§ 10^{-3}M	»	2.5	0.22	9
		§ 10^{-3}M	»	2.4	0.33	14
		§ 10^{-3}M	»	3.5	1.2	34
	Methyl green	2×10^{-3}M	»	3.5	1.6	46
		§ 2×10^{-3}M	»	2.4	1.1	46
		§ 2×10^{-3}M	»	2.4	1.3	54
Rat	Congo red	10^{-3}M	Antigen	1.8	0.99	55
		§ 10^{-3}M	»	1.8	0.98	54
		10^{-3}M	Compd. 48/80	1.5	0	0
		§ 10^{-3}M	»	1.5	0.12	8
	Trypan blue	§ 10^{-3}M	Antigen	1.4	0.72	51
		§ 10^{-3}M	Compd. 48/80	0.85	0	0
	Methyl orange	2×10^{-3}M	Antigen	0.74	0.45	61
		§ 2×10^{-3}M	»	1.4	1.2	86
		2×10^{-3}M	Compd. 48/80	0.89	0.50	56
		§ 2×10^{-3}M	»	0.85	0.85	100
	Sodium alizarin sulphonate	10^{-3}M	Antigen	0.90	0.07	8
		§ 2×10^{-3}M	»	1.4	0.78	56
		10^{-3}M	Compd. 48/80	0.66	0	0
		§ 2×10^{-3}M	»	0.85	0	0
	Rhodamine B	10^{-3}M	Antigen	0.74	0.94	127
		10^{-3}M	»	1.7	1.3	76
		10^{-3}M	Compd. 48/80	0.89	0.73	82
	Methyl green	2×10^{-3}M	Antigen	0.90	2.1	233
		2×10^{-4}M	»	1.7	1.4	82
		2×10^{-3}M	Compd. 48/80	0.66	2.1	318

§ Tissue washed after incubation with inhibitor.

The first four are acid and the last two basic dyes. Table V summarizes the observations on the effects of dyes upon histamine release in guinea pig and rat lung pieces. In guinea pig lung, anaphylactic histamine release was appreciably inhibited (in a concentration of 10^{-3} M to 2×10^{-3} M) by congo red, trypan blue and methyl orange, the effect was less pronounced when the tissue had been washed after incubation with the dyes, while in the case of rhodamine B and methyl green the inhibition continued unabated even after washing. Sodium alizarin sulphonate had very little effect in guinea pig tissue. Unlike the other enzyme inhibitors used, the dyes differed somewhat in their effects on guinea pig and on rat tissue. In rat, congo red and trypan blue were as effective in inhibiting anaphylactic histamine release as in guinea pig. The effect of methyl orange in rat was less pronounced but sodium alizarin sulphonate was much more effective. The most striking difference between the two species was seen with the two basic dyes rhodamine B and methyl green. While both inhibited histamine release in guinea pig, they were quite ineffective in rat; in fact, methyl green caused a release of histamine in the latter. It will be seen from Table V that despite this difference between the two species, the effect of the dyes on anaphylactic and compound-48/80-induced histamine release was much the same.

Discussion

Histamine release starts at its maximal rate within a few seconds after exposure of the tissue to the antigen and is complete in about 8 min. Since the contact with the antigen leads immediately to disruption of the mast cells, the initial rapid rate is to be expected. It appears unlikely, however, that the relatively slower subsequent rise of histamine value in the incubation fluid is due to the time required for diffusion of released histamine into that fluid, as was suggested by SCHILD (1939). It seems more probable to the author that the time taken for diffusion of the antigen, with its high molecular weight, into the deeper parts of the tissue, accounts for the slower release in the later stage of the reaction. This view is supported by the observation that the mast cell counts of the tissue show a progressive decrease up to 8 min, coinciding with the release of histamine (BORÉUS and CHAKRAVARTY 1960). When incubation was prolonged, the histamine concentration of the incubation fluid, after attaining its maximum value, decreased progressively. The possibility of enzymatic degradation was investigated. SCHAYER's (1956) findings indicate that three enzymes are concerned in the metabolism of physiological amounts of histamine in mammals: acetylating enzyme (which is considered to be of minor importance), diamine oxidase, and a third enzyme referred to as histamine metabolizing enzyme II. The latter two enzymes are believed to be equally effective in the guinea pig. Histamine metabolizing enzyme II is probably a composite of methylating and oxidative enzymes — the methyl

histamine possibly being further metabolized by monoamine oxidase. In the present experiments neither aminoguanidine nor iproniazid, which inhibit histaminase and monoamine oxidase respectively, prevented the decrease of histamine values on prolonged incubation; and hydrolysis of the solution did not increase its free histamine content, as would be expected in case of acetylation. Although phenol, 1 : 500, had no effect, the degradation might nevertheless be due to some bacterial action unaffected by this concentration of phenol; it might also be due to formation of methyl histamine. The problem requires further investigation.

The observations on histamine release from different tissues of guinea pig show that, with certain exceptions, the fraction of histamine released in anaphylaxis is similar irrespective of variable initial histamine content. — The most striking exception was the intestine which combined a high total histamine content with very poor release. An explanation for this discrepancy could be that a large fraction of histamine in the intestine is outside the mast cells. The mast cell population of guinea-pig intestine is, however, fairly high, but they are very resistant to disruption when exposed to antigen (Boréus and Chakravarty 1960). It is conceivable that the antigen-antibody reaction does not occur in the intestinal mast cells; or, after it has occurred, there is a lack of some essential constituent necessary for the process leading to their disruption.

The effects of concentration of antigen, temperature and pH point to an enzymatic process in anaphylactic histamine release, as suggested previously by Mongar and Schild (1957, 1958). The optimal pH in the present experiments was between 7 and 8.5, beyond which there was a rather sharp decrease in histamine release. These results were obtained using veronal buffer and did not differ essentially from those of Mongar and Schild (1958), who used phosphate or bicarbonate buffer. The effect of temperature, pH etc. on histamine release in rat was not investigated but observations made at this laboratory (Högberg and Uvnäs 1957, 1960) show that the degranulation of mast cells produced in the rat by compound 48/80 and in anaphylactic reaction is similarly dependent on temperature and pH. Thus both in guinea pig and rat an irreversible change, probably inactivation of an enzyme, occurs at 45° C, preventing subsequent histamine release at 37° C.

The effect of lack of oxygen and calcium on histamine release was investigated in guinea pig and rat. A striking difference was observed between the two species in regard to oxygen requirement. While anaphylactic histamine release was dependent on oxygen in guinea pig, lack of oxygen had no effect on histamine release in rat, either in anaphylaxis or in response to compound 48/80. The histamine release induced by compound 48/80 in perfused cat paw is also independent of oxygen, as reported previously (Chakravarty, Högberg and Uvnäs 1959); so is mast cell degranulation in rat by compound 48/80 or antigen-antibody reaction (Högberg and Uvnäs 1960). The difference in the

oxygen requirements of guinea pig and rat raises the question of how the two processes differ and at what stage oxygen may be required. There are indications of other differences in the histamine release processes of the two species (see below); but an answer to this question must await further investigation. If anaphylactic histamine release involves an energy-requiring process, it can apparently be maintained anaerobically in rat.

It has been shown that calcium is a requisite for anaphylactic histamine release both in guinea pig and rat and for compound-48/80-induced histamine release in rat. Histamine release inhibited by EDTA was largely restored when calcium chloride alone was supplied to tissues suspended in buffered 0.85 per cent NaCl solution containing EDTA and antigen. This suggests that calcium may be essential for the antigen-antibody reaction which initiates the anaphylactic reaction. Since histamine release elicited by compound 48/80 in the rat is also dependent on calcium, however, it seems more likely that calcium may be required at a later stage of the reaction — possibly as an activator of some enzyme involved in the process.

The blocking of histamine release by a number of inhibitors throws some light on the details of the process. The polymeric substances, hip-seed polysaccharide, Pk 11, Pk 312 and polyphloretin phosphate are fairly nonspecific enzyme inhibitors. However, the action of these substances of high molecular weight is probably at the cell surface; this assumption suggests that the activation of an enzyme on the surface of the mast cell leads to release of histamine.

Of the other enzyme inhibitors used, iodoacetate (DICKENS 1933), and allicin (WILLS 1956) react with sulphydryl groups of enzymes. — Both inhibitors were effective in blocking histamine release in guinea pig. Although the sulphydryl group is rapidly alkylated with iodoacetate, it may also combine with amino groups (MICHAELIS and SCHUBERT 1934); and allicin may react with some non-sulphydryl enzymes. Specific inhibition of the sulphydryl group is, however, indicated by the fact that glutathione and cysteine partially restore the histamine release. Iodoacetate was equally effective in inhibiting histamine release from the rat; and it has previously been shown that allicin blocks the degranulation of rat mast cells, the reaction being reversible in the presence of glutathione (HÖGBERG and UVNÄS 1958). In this respect, therefore, their action in anaphylactic and compound-48/80-induced histamine release in rat is similar to that in guinea-pig anaphylaxis.

Three amino group reactors — 2 : 4-dinitrofluorobenzene (DNFB), phenylisocyanate, and acetic anhydride — were effective inhibitors of histamine release. The specificity of the reaction should, however, be considered. DNFB may also combine with sulphydryl and phenolic hydroxyl groups, and phenylisocyanate may also react with sulphydryl groups (PUTNAM 1953); acetic anhydride, however, has been shown by HÖGBERG and UVNÄS (1957) to produce, in their experiments with rat tissue, specific acetylation of the amino

group. Hence the effect of the three inhibitors is most likely to be due to reaction with the amino group of enzyme protein.

The inhibition of histamine release by ethyl alcohol is not due to an irreversible change in the tissue since the release is unimpaired if the inhibitor is washed out before addition of antigen. It is effective in rats against histamine release induced by antigen or compound 48/80. Since these agents apparently release histamine in the rat by the same process, ethyl alcohol probably acts at a stage beyond the antigen-antibody reaction by a reversible interference with enzyme activation.

The inhibitory action of organic dyes on histamine release was not confined to any special groups; each of the six dyes tested showed some activity, either in guinea-pig or in rat tissue. Their effects nevertheless differed in the two species. In guinea pig, acid and basic dyes were alike. Both basic triphenylmethane dyes were effective in inhibiting histamine release, as were the acid azo dyes, congo red, trypan blue and methyl orange. In rat, however, neither of the two basic dyes was effective; in fact, methyl green released histamine in rat lung. But all acid dyes tested — viz. the three azo dyes and an anthraquinone dye, sodium alizarin sulphonate — inhibited histamine release; the inhibition by alizarin being much more pronounced than in guinea pig.

The striking difference in the effects of the two basic dyes suggests that the process of histamine release may differ in the two species. This view is further supported by the variance in oxygen dependency, for only in guinea pig does histamine release depend on oxidative metabolism. However, the enzyme inhibitors tested were as effective against histamine release in guinea pig as in rat, as was also the case with ethyl alcohol and the azo dyes. It follows that, this difference notwithstanding, the mechanisms in the two species must be largely alike.

It may be noted that though there is some difference between the two species, the processes of histamine release elicited in rat by antigen and by compound 48/80 are very similar in their dependence on calcium, their independence of oxygen, and their response to enzyme inhibitors, ethyl alcohol and organic dyes; these factors suggest that the same enzyme system is activated in anaphylaxis and by compound 48/80.

The investigation is not sufficiently comprehensive for conclusions as to the mode of action or molecular specificity of the dyes. Since they are equally effective against histamine release induced by anaphylaxis and by compound 48/80, they seem to act upon some tissue constituent rather than the antigen-antibody reaction. The acid and basic organic dyes have been shown to inhibit bacterial dehydrogenases and other enzymes (Quastel and Wheatley 1931; Quastel and Yates 1936). The inhibitory effects of the dyes on anaphylactic reaction could be due to blockage of the enzyme system involved in anaphylaxis. Since either acid and basic dyes are effective in guinea pig, it is likely that both acid and basic groups of the enzyme protein are essential for its activity in this species.

Summary

1. A report is presented of *in vitro* studies on anaphylactic histamine release in guinea pig and rat, and histamine release induced by compound 48/80 in rat.

2. The time course, the release from different tissues, and the effects of antigen concentration, temperature, and pH were investigated in guinea pig. Anaphylactic histamine release was dependent on temperature and pH. The histamine value of the incubation fluid decreased progressively on prolonged incubation. Loss of free histamine could not be prevented by aminoguanidine, iproniazid (marsilid) or phenol (1 : 500).

3. Anaphylactic histamine release occurred normally in rat tissue in the absence of oxygen, while that in guinea pig tissue was dependent on oxygen. Calcium was necessary for histamine release in both species.

4. Polymeric substances — e. g. hip-seed polysaccharide, polymers of salicylic acid and o-aminobenzoic acid and polyphlorein phosphate — inhibited histamine release both in guinea-pig and rat tissue. Other enzyme inhibitors, — iodoacetate and allicin, which react with — SH groups, and dinitrofluorobenzene, phenylisocyanate and acetic anhydride, which react with —NH_2 groups — were equally effective for inhibiting histamine release in both species; so, also, were ethyl alcohol and acid organic dyes, especially azo dyes. However, two basic dyes of the triphenylmethane group inhibited histamine release in guinea pig but not in rat.

5. The observations point to the mediation of histamine release in anaphylaxis by an enzyme system with essential —SH and —NH_2 groups. Apparently, there is some difference in the processes of anaphylactic histamine release in guinea pig and rat, though in many ways they are similar. In the rat histamine release produced by anaphylactic reaction is mediated by the same or similar mechanisms as in that induced by compound 48/80.

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The Occurrence of a Lipid-Soluble Smooth-Muscle Stimulating Principle ('SRS') in Anaphylactic Reaction

By

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Abstract

CHAKRAVARTY, N. *The occurrence of a lipid-soluble smooth-muscle stimulating principle ('SRS') in anaphylactic reaction.* Acta physiol. scand. 1960. *48.* 167—177. — When tissues from sensitized guinea pigs are incubated with antigen, there is a diffusion of histamine and a lipid-soluble, smooth-muscle stimulating principle ('SRS') into the incubation fluid.. 'SRS' is soluble in ether and other organic solvents and is of acid nature. The time curves of histamine and 'SRS' are similar except for the slower appearance of 'SRS' in the first few minutes. There is a close correlation between the amounts of histamine and 'SRS' obtained from different tissues. Moreover, with increasing concentrations of antigen the amounts of both histamine and 'SRS' rise proportionally. The observations are compatible with the view that the two smooth-muscle stimulating substances may originate from a common source.

It was shown in an earlier communication that a lipid-soluble, smooth-muscle stimulating principle appears from perfused cat paw after the injection of compound 48/80[1] (CHAKRAVARTY, HÖGBERG and UVNÄS 1959). The present report is based on a smooth-muscle stimulating principle, which occurs in the incubation fluid following *in vitro* anaphylactic reaction in isolated guinea pig tissue. This is probably the same principle which KELLAWAY and TRETHEWIE (1940) first reported to be released in anaphylactic reaction from perfused guinea pig lung. Its chemical nature is unknown and knowledge of its biological properties is fragmentary.

[1] Condensation product of p-methoxyphenethylmethylamine with formaldehyde.

The name slow reacting substance given to it refers only to one of its biological properties, *viz.* a contraction of smooth muscle. A number of substances, especially polypeptides and lipid-soluble acids, have a similar effect on smooth muscle and some of them have been given the same name (FELDBERG, HOLDEN and KELLAWAY 1938, TRETHEWIE 1942, GADDUM 1955, VOGT 1956, 1957). It is necessary, therefore, to designate the additional smooth-muscle contracting principle of anaphylaxis by a more specific name with respect to its chemical and biological properties in order to distinguish it from the other substances. However, since its chemical nature is not yet known, we have retained the old name with reservations ('SRS') to avoid further confusion.

Since 'SRS' is one of the products of anaphylactic reaction, it was thought that an investigation into the quantitative relation between the occurrence of histamine and 'SRS' might throw some light on the process. A method of assaying 'SRS' was recently developed at this laboratory (CHAKRAVARTY 1959), thus permitting a study of the quantitative relationship between histamine and 'SRS'. The present report, comprising the results of such an investigation, shows a close parallelism between the occurrence of histamine and 'SRS' in anaphylactic reaction.

Material and Methods

Guinea pigs weighing 250 to 350 g were sensitized by two injections of 0.1 g crystalline egg albumin subcutaneously and intraperitoneally within a week. They were used for anaphylactic reaction in the isolated organs 4 to 14 weeks after the first injection.

Anaphylactic reaction was produced *in vitro*, using a modification of the method of UNGAR and PARROT (1936) as described previously (CHAKRAVARTY 1960). The lungs — cut into small pieces — were used for all experiments except those in which different tissues were specifically investigated. The tissues were incubated in Tyrode solution with antigen (1 mg/ml) at 37° C for 10 min; the time and concentration of antigen were varied only when their effect was under study. During the incubation, there was little or no spontaneous histamine release. When a detectable release did occur, the relevant value was deducted to get the anaphylactic histamine release. 'SRS' was not released in the absence of antigen. Using duplicate controls, the amounts of histamine and 'SRS' which appeared in anaphylactic reaction were found to be sufficiently uniform (see below) to permit evaluation of the effect of different experimental procedures. For further details of the procedures see CHAKRAVARTY (1960).

Residual histamine in the tissue was determined by heating the pieces in water (10 ml/g) plus 1 N HCl (2 ml/g) at boiling point for three minutes. The extract was neutralized and brought up to suitable volume for testing on guinea pig ileum.

Histamine was assayed on atropinized (1.5×10^{-6} M atropine sulphate) guinea pig ileum. The values are expressed as histamine base.

'SRS' was also assayed on guinea pig ileum in the presence of atropine (1.5×10^{-6} M) and an antihistaminic (10^{-7} M to 10^{-6} M mepyramine). The quantitative values for 'SRS' are expressed in units, a unit being the amount of 'SRS' which appeared in the incubation fluid from 10 mg lung tissue in one experiment. Preparation of standard and the method of assay have been detailed in a separate communication (CHAKRAVARTY 1959), and it has been shown that 'SRS' can be assayed on guinea pig ileum with relatively narrow margins of error.

The presence of 'SRS' did not interfere with histamine assays because of the relatively higher sensitivity of the gut to histamine in the test solution as compared to 'SRS'. The small volume needed for testing histamine produced no 'SRS' response. The samples were kept in contact with the gut for 15 to 20 seconds for histamine assay and 1.5 to 2.5 min for 'SRS' assay.

The content of egg albumin in the test samples applied to the gut had no effect on histamine and 'SRS' contraction.

'SRS' was tested either in the original incubation fluid or following extraction in alcohol and ether. The extraction procedures were as follows: The incubation fluid was freeze-dried and extracted with 80 per cent ethyl alcohol. The alcohol extract was dried under reduced pressure in a nitrogen atmosphere at a bath temperature of 50 to 55° C. The dried residue was dissolved in water and tested on guinea pig ileum. In ether extraction, following initial alcohol extraction and drying, the sample was acidified with dilute hydrochloric acid (0.1 to 1 N) and shaken with ether. 'SRS' passed over to the ether layer and could be re-extracted to aqueous phase by shaking with alkaline water. The alkaline water layer was then neutralized and freeze-dried. It was subsequently dissolved in a relatively small volume of water to give an isoosmotic solution, and tested on guinea pig ileum.

Results

When pieces of lung from sensitized guinea pig were incubated with antigen, both histamine and 'SRS' could be detected in the incubation fluid. It was possible, therefore, to study the relationship between the occurrence of the two substances under different experimental conditions. Furthermore, 'SRS' could be thus produced in sufficient amounts to permit some preliminary observations on its chemical properties.

Chemical properties of 'SRS'

'SRS' can be completely extracted with 80 per cent ethyl alcohol. It is partly soluble in methyl alcohol, n-propanol, and water-saturated n-butanol. After acidification 'SRS' passes over to ether and other organic solvents, viz. ethyl acetate, methyl ethyl ketone, chloroform and petroleum ether (see Table I).

When 'SRS' in aqueous solution at various pH is shaken with ether a characteristic distribution pattern is obtained, as shown in Fig. 1. — Alcohol-extracted 'SRS' was dissolved in water and divided into several equal parts. One of these was used to determine the activity in the original sample. To the others a few drops of dilute hydrochloric acid (0.1 to 2 N) were added to obtain different pH between 2 and 7.5, and they were agitated with equal volumes of ether in a mechanical shaker. After 10 minutes the ether and aqueous layers were allowed to separate. 'SRS' in the aqueous phase could be tested directly after evaporation of the ether and adequate neutralization. 'SRS' in the ether phase was tested after re-extraction with alkaline water. It will be seen from Fig. 1 that between pH 2 and 4, 65—73 per cent of the 'SRS' activity was in the ether layer, and only 27—35 per cent was in the aqueous layer. An equal

Table I. Solubility of 'SRS' in organic solvents

Solvent	Recovery of 'SRS' from solvent	'SRS' in residue after extraction
80 % Ethyl alcohol	+	0
Ethyl alcohol (Abs.)	0	+
Acetone	0	+
Methyl alcohol	+	+
n-Propanol	+	+
n-Butanol (Water sat.)	+	+
Ether	+	0
Ethyl acetate	+	0
Methyl ethyl ketone	+	0
Chloroform	+	+
Petroleum ether (Skellysolve B)	+	+

The first five solvents were used directly for 'SRS' extraction from freeze-dried material; the last five were used following extraction with 80 % ethyl alcohol, drying and acidification with dilute HCl. n-Butanol was used in both ways.

distribution between the ether and water phases was found between pH 4 and 5. Beyond pH 5 there was a sharp decrease of 'SRS' in the ether layer, with a corresponding rise in aqueous layer. At pH 6.1, 95 per cent of 'SRS' activity was already present in the aqueous fraction and only 5 per cent in the ether fraction. The pH distribution of 'SRS' was similar when phosphate buffer was used for the aqueous phase and shaken with a solution of 'SRS' in ether. It may be noted here that the percentual values in ether and water had to be calculated from the total amount of 'SRS' recovered from the two phases. This constituted 60 to 90 per cent of the original activity in different experiments; 10 to 40 per cent of 'SRS' activity was apparently lost somewhere during the experimental procedure.

When alcohol-extracted 'SRS' was acidified with dilute hydrochloric acid, it readily passed over to organic solvents; it could then be tested on guinea pig ileum following re-extraction from the solvent in alkaline water. As shown in Fig. 2, the extraction was highest with methyl ethyl ketone, and lowest with petroleum ether (skellysolve B). Apparently there was some loss of activity during extraction. In the case of solvents like chloroform and petroleum ether the extraction was also evidently incomplete, and 'SRS' could be detected in the residue after extraction (see Table I).

'SRS' is unstable at room temperature. Between pH 7.5 and 9.5 about one-half of the activity disappeared in 24 hours. The inactivation was less pronounced at lower than at higher pH as shown in Fig. 3. Inactivation could

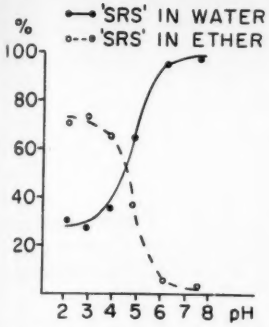

Fig. 1. Distribution of 'SRS' between ether and water between pH 2 and 8. As the pH decreases the amount of 'SRS' in the aqueous phase falls and that in the ether phase rises.

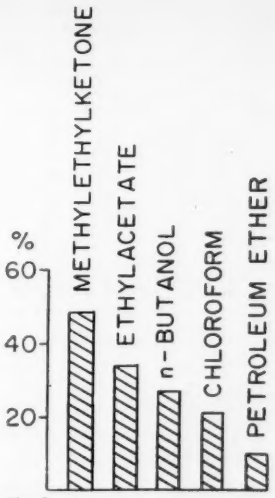

Fig. 2. Extraction of 'SRS' in organic solvents following acidification. The percentage of original activity recovered from the solvents is shown in the figure.

not be prevented by keeping the solution in nitrogen atmosphere or by reducing substances; nor did exclusion of bacterial action by boiling prevent inactivation.

Alcohol-extracted 'SRS', on boiling for 15 min in an aqueous solution, lost about one-tenth of its activity. Boiling with alkali (one-tenth volume of 1 N NaOH) caused no further inactivation, but boiling with dilute hydrochloric acid in the same proportion destroyed about one-half of the activity. — In this respect its property is somewhat similar to that of Darmstoff (VOGT 1957). — It was also observed that boiling with a reducing substance like glutathione (10^{-2} M), or sodium dithionite (5.7×10^{-3} M) destroyed over two-thirds of the activity; boiling with an oxidizing agent, periodic acid (4.4×10^{-3} M), had a similar but less pronounced effect. These findings are illustrated in Fig. 4.

Histamine and 'SRS' in Anaphylactic Reaction

When several uniform samples of sensitized lung tissue were exposed to antigen, the duplicate tests showed relatively small differences in their histamine and 'SRS' values (see Table II). It was therefore possible, by treating some of the samples with inhibitors, or by other procedures, to determine their effect on the appearance of histamine and 'SRS'.

Time course

The time curve for the appearance of histamine and 'SRS' was determined in three experiments by incubating a relatively large sample of sensitized tissue with antigen and withdrawing samples at various intervals. In the first few

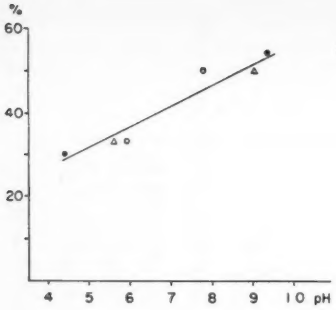

Fig. 3. Percent inactivation of 'SRS' in 24 hours at room temperature (20—22° C). Results of 3 experiments. Note more inactivation at higher pH.

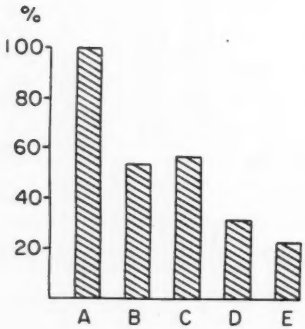

Fig. 4. Inactivation of 'SRS' on boiling in a water bath for 15 min under different conditions:
A = with 1/10 vol. 1 N NaOH (at pH 12.3),
B = with 1/10 vol. 1 N HCl (at pH 1.1),
C = with 4.4×10^{-3} M periodic acid ($HIO_4 \cdot 2H_2O$),
D = with 10^{-2} M reduced glutathione and
E = with 5.7×10^{-3} M sodium dithionite ($Na_2S_2O_4$).

The values are expressed as percentage of activity of a sample boiled at neutral pH.

minutes 'SRS' appeared at a slower rate than histamine. This was very evident when the histamine and 'SRS' values at one or two minutes were expressed in per cent of their maximum values in the experiments. At one minute 38 to 62 per cent of the total released histamine appeared in the incubation fluid,

Table III. Histamine and 'SRS' from different tissues of the guinea pig following anaphylactic

Expt.	Aorta		Lung		Uterus		Heart	
	H	'SRS'	H	'SRS'	H	'SRS'	H	'SRS'
1	6.8	467	3.6	208	0.60	26	0.53	21
2	2.7	250	1.6	189	1.7	103	0.36	33
3	3.8	346	1.6	170	0.77	70	0.62	45
4	2.7	328	0.95	162	1.6	228	0.68	108

H = histamine μg/g tissue. 'SRS' expressed in units/g tissue.

Table II. Histamine and 'SRS' in duplicate samples

Expt.	Sample 1		Sample 2	
	H	'SRS'	H	'SRS'
1	2.9	70	2.4	56
2	4.7	160	4.5	129
3	1.3	115	1.2	109
4	3.3	396	3.2	491
5	4.7	123	4.7	100

H = histamine μg/g tissue. 'SRS': expressed in units/g tissue. In Expt. 1 & 2 the tissues (guinea pig lungs) were incubated with antigen for five minutes and in Expt. 3 to 5 for 10 minutes.

the corresponding figures for 'SRS' being only 0 to 2 per cent; at two minutes the respective values were 65 to 76 per cent for histamine and 0 to 24 per cent for 'SRS'. As shown in Fig. 5, the slower rate for 'SRS' was noticeable up to 4 min, during which there was a fairly abrupt release of histamine. From 8 to 32 min the two substances had a more or less parallel course. When incubation was continued further, both histamine and 'SRS' values progressively decreased.

The reduction of 'SRS' activity on prolonged incubation could be due to its enzymatic or bacterial degradation. Since, however, both histamine and 'SRS' values fell after reaching the peak at about the same time, and in view of their basic and acid natures respectively, the possibility of their conjugation was studied. In one experiment 1.7 μg/g histamine was released in 10 min following the addition of antigen. When incubation was continued for four hours the histamine value of the incubation fluid was reduced to 0.83 μg/g. 'SRS' in the incubation fluid was 144 units/g at 10 min, and at four hours could not be detected. On extraction of the tissue with 80 per cent al-

reaction in vitro

Trachea		Skin		Intestine (Jejunum)		Liver		Striated muscle	
H	'SRS'	H	'SRS'	H	'SRS'	H	'SRS'	H	'SRS'
—	—	—	—	—	—	—	—	—	—
—	—	0.14	22	0.12	0	0.03	0	—	—
1.7	117	0.27	5	0.34	0	0	0	0.09	4
1.4	186	0.54	26	0.09	0	0.05	0	0.09	0

cohol 36 units/g 'SRS' were obtained at 10 min, and no 'SRS' was detectable at four hours, although both extracts had about equal amounts of histamine. Hydrolysis of the incubation fluid at four hours with hydrochloric acid did not increase the free histamine value. These findings conflict with the assumption of conjugation between histamine and 'SRS' in the incubation fluid or the tissue. The cause of inactivation of 'SRS' on prolonged incubation remains to be elucidated.

Histamine and 'SRS' from different guinea pigs

It is well known that guinea pigs may vary considerably in the anaphylactic histamine release, depending on the initial histamine content of the tissues and the degree of sensitization. This fact provided an opportunity to study the relationship between the appearance of histamine and 'SRS' in anaphylactic reaction. Isolated lung pieces from a batch of 5 guinea pigs were separately incubated with antigen 40—60 days after the first sensitizing dose of antigen. The histamine release varied from 1.4 to 6.9 μg/g, the corresponding figures for 'SRS' varying from 61 to 420 units/g. The correlation between the two substances was striking, as shown in Fig. 6. The animals chosen for this experiment were of about the same weight and from the same litter. If widely differing guinea pigs and experimental conditions are employed, such a correlation may be lacking. It should be emphasized that the time of incubation is an important factor, especially in view of the slower rate of appearance of 'SRS' in the early stage of anaphylactic reaction. The proportion of histamine to 'SRS' may vary also with the age and strain of the animal. These factors might account for the lack of correlation between the output of 'SRS' and that of histamine from perfused guinea pig lungs, as reported by KELLAWAY and TRETHEWIE (1940).

Histamine and 'SRS' from different tissues

In order to study the correlation further, different tissues of sensitized guinea pigs with widely differing amounts of releasable histamine were incubated with antigen. A wide range of histamine release levels showed a marked correlation to 'SRS', whether tested directly in the incubation fluid (see Table III) or following ether extraction (see Fig. 7).

Effect of concentration of antigen

Further evidence of parallelism between the occurrence of histamine and of 'SRS' was obtained when lung pieces from sensitized guinea pigs were incubated with different concentrations of antigen (see Fig. 8). Neither histamine nor 'SRS' appeared at an antigen concentration of 10^{-7}; above this concentration the values for both rose proportionally, the two curves running quite parallel. The results in a second experiment were similar.

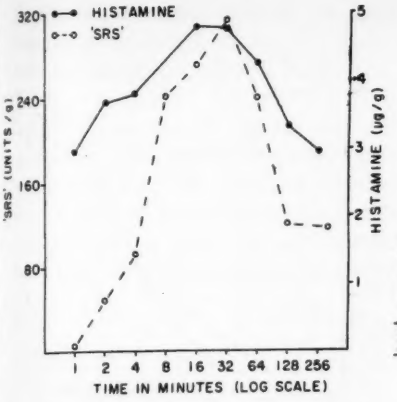

Fig. 5. Time course of the appearance of histamine and 'SRS' from guinea pig lung pieces in anaphylactic reaction *in vitro*.

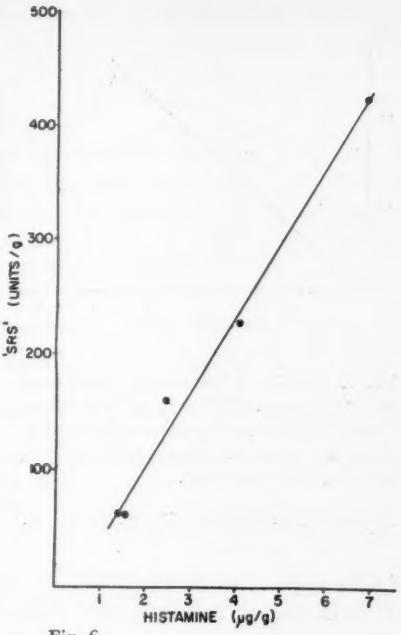

Fig. 6. Correlation between histamine and 'SRS' following *in vitro* anaphylactic reaction in lung pieces from 5 different guinea pigs. Histamine was tested in Tyrode solution, 'SRS' in alcohol extract.

Fig. 6

Discussion

The inactivation of 'SRS' by heating with acid, and its resistance to alkali, have a superficial resemblance to the behaviour of Darmstoff, which is also a lipid-soluble acid (VOGT 1957); but Darmstoff produces only a slight effect on guinea pig ileum and differs in its chromatographic behaviour from 'SRS' (to be published). Prostaglandin is also a lipid-soluble acid (EULER 1936). However, testing of the differential sensitivity of prostaglandin[1] and 'SRS' on guinea pig ileum and rabbit duodenum (unpublished observation) demonstrated that they were different substances. Unsaturated fatty acids isolated from plasma (GABR 1956) and rabbit iris (AMBACHE 1959), or formed in egg yolk by cobra venom (VOGT 1956), have been shown to produce slow contraction of guinea pig intestine. A few other lipid-soluble, biologically active substances have been described, but their chemical and biological natures are rather ill-defined. 'SRS' probably belongs to the general group of lipid-soluble acids (VOGT 1958) but is not identical with any of the known substances.

The time curves for histamine and 'SRS' are similar except for a slower appearance of 'SRS' in the first few minutes. A similar delay was observed

[1] My thanks are due to Prof. Sune Bergström and to Dr. Rune Eliasson for the supply of prostaglandin.

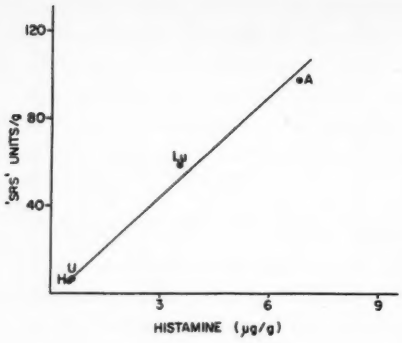

Fig. 7. Correlation between the appearance of histamine and 'SRS' from different tissues of guinea pig in anaphylactic reaction *in vitro*. Histamine tested in Tyrode solution, 'SRS' following ether extraction.

A=Aorta, Lu=Lung, U=Uterus, H=Heart.

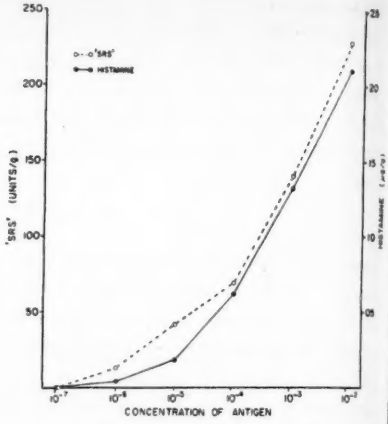

Fig. 8. Effect of concentration of antigen (w/v) on the appearance of histamine and 'SRS' from guinea pig lung pieces in anaphylactic reaction *in vitro*.

in the appearance of 'SRS' induced in perfused cat paw by compound 48/80 (CHAKRAVARTY, HÖGBERG and UVNÄS 1959). In both instances the delay might well be explained by a high molecular weight of 'SRS', with low diffusion rate, though other explanations are also possible. It may be noted in this connection that heparin, which has a relatively large molecule, appears in canine plasma at a slower rate than histamine in the first few minutes of anaphylactic reaction (PATON 1951). If incubation of sensitized tissues with antigen was continued for prolonged periods, both histamine and 'SRS' values gradually fell. Aminoguanidine and iproniazid did not prevent this decrease. The similar time course suggested the possibility of conjugation between basic histamine and acid 'SRS'. Yet acid hydrolysis of the incubation fluid did not increase its free histamine content. Comparative plotting of the amounts of histamine and 'SRS' in the incubation fluid and those remaining in the tissue at 10 min and four hours showed, moreover, that some histamine and 'SRS' had been inactivated by prolonged incubation. Bacterial and/or enzymatic degradation of both histamine and 'SRS' seems to be the most plausible explanation of their inactivation, but this point requires further elucidation.

Histamine and 'SRS' appeared in proportional amounts in anaphylactic reaction *in vitro* from the lungs of different guinea pigs of the same strain and weight. The same correlation was observed over a wide range when different tissues from sensitized guinea pigs were incubated with antigen. RILEY and WEST (1955) and MOTA *et al.* (1956) found a close correlation between histamine release in rats induced by compound 48/80 and mast cell population,

indicating that the releasable histamine is located in the mast cell. Histamine release in anaphylactic reaction in the guinea pig is also associated with mast cell degranulation (MOTA 1958). The striking correlation between the occurrence of histamine and 'SRS' in the present experiments could, therefore, be explained on the assumption that 'SRS' originates from the mast cells. This is further indicated by our experiments in which the tissues were examined with respect to their mast cell population after incubation with antigen; there was a marked reduction in the number of mast cells following anaphylactic reaction which led to the appearance of histamine and 'SRS' in the incubation fluid (BORÉUS and CHAKRAVARTY, to be published).

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Interhippocampal Impulses

II. Apical Dendritic Activation of CA1 Neurons

By

PER ANDERSEN

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Abstract

ANDERSEN, P. *Interhippocampal impulses. II. Apical dendritic activation of CA1 neurons.* Acta physiol. scand. 1960. *48.* 178—208. — This work is an anlysis of the commissural response of the field CA1 of the hippocampus, a surface positive wave with a spike, followed by a negative wave. The major, later part of the surface positive wave was recorded negative in the layer of apical dendritic shafts. This deep negative wave is interpreted as a summated excitatory postsynaptic potential. It seems to have a causal relationship to the production of the spike. The spike most often showed its shortest latency in the apical dendritic shaft layer, increasing in both directions along the dendritic tree. The speed of propagation along the dendritic shaft was about 0.35 m/sec. The surface negative wave is confined to the basal layers and is probably partly due to the spread of the depolarizing wave along the dendrites and partly to the activity of alveus-near located neurons. In the CA1 apical dendritic membrane two mechanisms were found — one was responsible for the production of the deep negative wave — the other was responsible for the initiation and propagation of the spike along the dendrites.

In a previous work (ANDERSEN 1959) the commissural responses from the fields CA1 and CA3 of the hippocampus were described for the cat, rabbit and rat. The form of the potentials was in agreement with that reported by

CRAGG and HAMLYN (1957) for the rabbit. The distribution of the evoked potentials agreed with normal anatomical data (CAJAL 1911, LORENTE DE NÓ 1934) and recent experimental histological findings (BLACKSTAD 1956).

As stressed by EULER, GREEN and RICCI (1958), data gained from the study of activation of the relatively simple hippocampal cortex may also help the interpretation of activation of more complex parts of the cerebral cortex.

The termination of commissural fibres upon restricted parts of the dendritic tree of CA1 neurons (BLACKSTAD 1956) revealed a suitable substrate for an electrophysiological study of the activation pattern of the CA1 cortex by commissural impulses, especially the rôle of the dendrites in this activation. In addition, the special arrangement of the CA1 dendrites favours the study of a possible propagation of potentials along the apical dendrites.

The aim of the present investigation has been to study the activation of CA1 neurons by interhippocampal impulses. The possibility of spike production in the apical dendrites was of particular interest.

Material and Methods

Ten cats, 30 rabbits and 6 rats were used, all adult animals. Detailed description of the methods employed was given in an earlier article (ANDERSEN 1959).

The animals were anesthetized with urethane-cloralose, and conventional evoked potential techniques were used. The analysis of the potential was made by monopolar surface and depth recording using steel electrodes of 3—25 μ tip diameter; paired and repetitive stimuli; local application to the recording site of 2 per cent procaine, 6 per cent sodium pentobarbital and 0.1—1 per cent strychnine sulfate; anoxia; local and general cooling, and sectioning of various structures. The paired stimuli intervals were from 0 to 400 msec. The repetitive stimulating frequency was varied between 1/sec and 550/sec, given in periods from 40 msec to 30 sec. The drugs were locally applied on 2 × 2 mm pieces of filter paper. These were gently placed upon the hippocampal surface. The recording electrodes were introduced through a thin slit. Anoxia was induced by immobilization of the animals with decamethonium bromide (cats 0.1 mg/kg, rabbits 0.25 mg/kg) given intravenously, supplemented with smaller doses when necessary. The animals were artificially ventilated with pure oxygen under overpressure; and the oxygen was replaced by nitrogen for 1—30 min, usually 3—4 min. Local cooling was produced by irrigation of the pool with liquid paraffin of 8° C. When general cooling was used, the animals were first shaved and immobilized by decamethonium (doses as reported above). Then the animals were immersed to the neck in ice-water. The temperature was measured on the hippocampal surface, in the paraffin pool and in the rectum by thermocouples (tip diameter 1 mm). The electrocardiogram was recorded to control the pulse rate and any failure of the heart. The electrode locations were confirmed in reduced silver sections with small electrocoagulations as reference points (ANDERSEN 1956).

Terminology. When an electrode penetrates the cortex of the hippocampus from its ventricular surface it moves in the opposite direction to that used in comparable studies on the neocortex. It first punctures the subjacent cortical white matter, the alveus, — than the layer of basal dendrites, the stratum oriens, — subsequently the pyramidal cell body layer, the stratum pyramidale, — then the layer of the apical dendritic shafts,

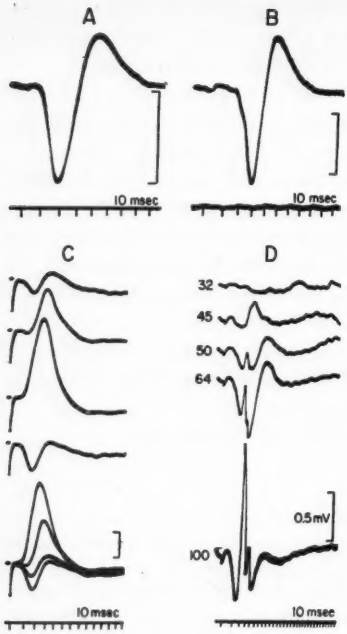

Fig. 1. CA1 commissural surface potential. — *A.* Usual form. — *B* in addition shows the early positive wave. — *C.* Gradual, spontaneous development from the usual pattern to a pure negative wave and back to the original pattern. Interval between records 5 sec. The lowest record shows the superimposition of the records above. — *D.* Effect of increasing stimulus strength, given in arbitrary units.
In all figures the records presented are from rabbit experiments, and obtained by monopolar recording. Negativity upwards.

the stratum radiatum, — and finally the layer of the terminal branches of the apical dendrites, the stratum lacunosum-moleculare.

Due to the peculiar anatomy of the hippocampus the term *surface* potential in this paper means that the electrode tip is on the alveus (ventricular surface) of the hippocampus. *Depth* potentials are recorded when the electrode is deep to the alveus. Somewhat paradoxically, the term *superficial* refers to events occurring near the hippocampal fissure, while *basal* refers to potentials recorded near the alveus.

Results

A. Pattern of responses

Surface recorded potentials. The CA1 commissural potential was recorded from CA1 following stimulation of the symmetrical contralateral point. Fully developed it consisted of a surface positive/negative wave with one or more spikes superimposed on the positive phase (Fig. 1) (ANDERSEN 1959). The polarity of the spikes was positive, negative, or diphasic positive/negative depending on the excitability of the cortex and the depth of the electrode. High cortical excitability and relatively large electrode tips favoured surface negativity of the spikes.

Although the CA1 commissural response usually was quite constant, some variation was occasionally noted. When the cortical excitability was low, or the stimulation intensity low, the spike might fail to show (Fig. 1). The level of cortical excitability was judged from the number and the magnitude of evoked spikes, the degree of amplitude variation of the surface negative wave and the increased probability of the occurrence of "spontaneous", sustained electrical afterdischarges. The most stable part of the potential was the surface positive wave, usually none of the other two components was seen unless the positive wave was present. Sometimes it showed two parts, an early small and a later great one (Fig. 1 B). The negative phase was absent in some experiments. On the other hand, it was prominent when recorded in periods of high excitability. The entire response was then often purely negative. However, in such circumstances a clear development from the normal picture was seen with an increasing negative phase encroaching more and more upon the positive wave (Fig. 1 C). When present, the pure negative response was seldom recorded for periods longer than 15 seconds, unless spontaneous sustained afterdischarges developed.

Stimulus threshold measurements demonstrated (Fig. 1 D) that the negative wave occurred at the same stimulus intensity as the positive wave, while the spikes appeared on stronger stimulation. Experiments with increasing stimulus intensity seemed to show that the surface positive component was the essential part of the CA1 commissural potential, necessary for the production of the spike.

Depth recorded potentials. Following single shock stimulation of the CA1 surface, a recording electrode penetrating the contralateral field CA1 parallel to the apical dendritic shafts (56 electrode tracks in 10 cats and 15 rabbits) revealed a typical change in the potential with increasing depth (Fig. 2 B and D). The positivity of the earlier part of this wave persisted throughout the depth of the CA1 cortex, showing its lowest amplitude in the stratum radiatum, *i. e.* the layer of the apical dendritic shafts. The rest of the diphasic surface positive/negative potential (B), changed in a typical manner in nearly all experiments when the electrode tip passed beyond 0.5—0.7 mm from the ependymal surface of the alveus. This depth corresponds to the pyramidal cell layer. The change consisted of a polarity reversal of the latter, major part of *the surface positive potential.* This was recorded as a negative wave in the layers of the CA1 cortex lying superficial to the pyramidal layer. Its maximal amplitude was usually obtained in the middle of the stratum radiatum, or sometimes, at the border between this layer and the stratum lacunosum-moleculare.

The amplitude of *the surface negative wave* was gradually diminished as the recording electrode approached the pyramidal layer, and usually no clear deflection corresponding to this component was recorded from points superficial to this stratum, *i. e.* near the hippocampal fissure. The main part of the

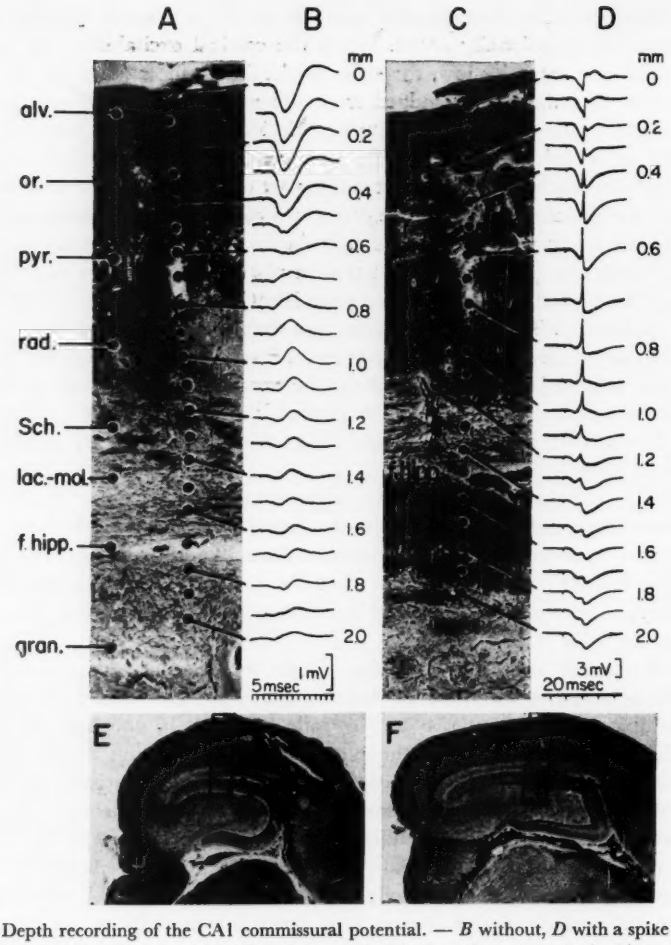

Fig. 2. Depth recording of the CA1 commissural potential. — *B* without, *D* with a spike superimposed. The photomicrographs in *E* and *F* are of sagittal sections through the hippocampus and indicate the site of the columns *A* and *C*, respectively. Abbreviations for all figures:

alv.	alveus
f. hipp.	hippocampal fissure
gran.	granule cell layer of the dentate area
hil. f. dent.	hilus of the dentate fascia
lac.-mol.	stratum lacunosum-moleculare
or.	stratum oriens
perf. path	perforant path
pyr.	pyramidal layer
rad.	stratum radiatum
Sch.	Schaffer collaterals

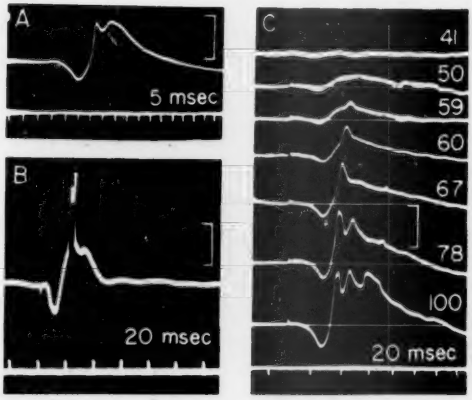

Fig. 3. Relation between the deep negative wave and the spikes as recorded from the layer of the apical dendritic shafts of CA1 in response to contralateral stimulation. — *A* shows one spike and *B* two spikes riding on the negative wave. *C*. Effect of increasing stimulus strength, given in arbitrary units. Voltage calibration 1 mV.

surface negative wave is therefore, probably due to activity in the basal layers of the cortex.

The spike was recorded with its maximal amplitude in, and slightly superficial to, the pyramidal cell layer (Fig. 2 D). When the spike was surface positive or diphasic it became negative 0.3—0.4 mm below the ependyma, corresponding to the layer of the basal dendrites of the pyramidal cells. The spike was recorded negative in all parts of the stratum radiatum, but its amplitude diminished rapidly near the molecular layer. Although the spike sometimes invaded this layer, it most often failed to do so, as judged by the reversal of its polarity at the border between the stratum radiatum and the molecular layer.

The commissural radiatum response. The spike latency measurements (see next section) suggested that the spike generation, following contralateral CA1 stimulation, took place in the layer of the apical dendritic shafts of the pyramidal cells. Therefore, closer attention was paid to the potential complex recorded from this region following symmetrical contralateral CA1 stimulation, "*the commissural radiatum response*". It consisted of a positive wave followed by a longer negative wave of greater amplitude with one or more spikes superimposed (Fig. 3). The initial positive wave varied in magnitude and was sometimes almost lacking. The negative wave had an amplitude as high as 1.5—2 mV and lasted from 25—60 msec when recorded with d. c. amplification. The duration of the rising phase varied with the stimulus strength from 4 to 11 msec. The decay was approximately exponential with a time constant of 16—25 msec with d. c. recording. The negative wave had its shortest latency

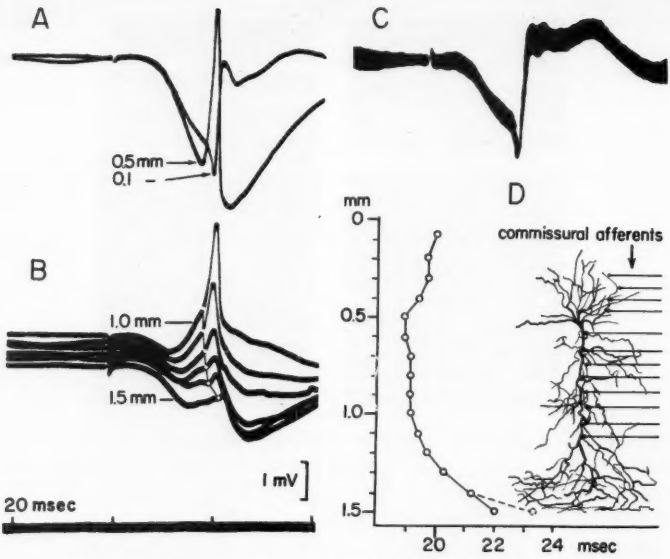

Fig. 4. Recording of spike latency at different depths of the CA1 cortex in response to a commissural volley. — *A*. Superimposition of records obtained 0.1 and 0.5 mm below ventricular surface to show latency difference. — *B*. Superimposition of records obtained every 0.1 mm from 1.0 to 1.5 mm below ventricular surface. The onset of the spike in each record is marked with a white point and these points are interconnected to show the increasing latency at increasing depths. — *C*. Superimposition of all *surface* records obtained simultaneously with the depth records shown in *A* and *B*. No spontaneous latency variation is seen. — *D*. Graph illustrating the variation of the spike latency at different depths. To the right a CA1 pyramidal cell is drawn to scale and at the correct level below the ventricular surface to facilitate comparison of the spike latency with morphological data.

and steepest rising limb at about 1.0 mm below the ventricular surface (Fig. 2 B, 4 B).

The positive and negative waves had the same stimulus threshold (Fig. 3 C). On increasing the intensity of the stimulus, the duration of the negative wave remained much the same, while the amplitude increased and the rising phase became steeper. Over a certain threshold, one or more spikes occurred "riding" on the negative wave. On further increase in the stimulus strength, the spikes increased both in magnitude and number, while their latencies decreased. The spikes seldom occurred on the falling phase of the negative wave, and then only on the earliest part of it. In the rabbit the spike latencies gathered into two groups, 13—19 and 24—35 msec (in cat one around 20 and one around 30 msec, in rat not sufficient data), using the usual CA1 electrode locations (interelectrode distance along the pathway approximately 25 mm

in rabbits). The spikes having the short latency were probably the result of direct activation, while those with the long latency probably were due to relay through ipsilateral CA3 neurons (see the following section).

B. Measurement of the spike latency

The latency of the spike was measured to the point where it departed from the deep negative wave, or — when the spike was diphasic — to the steepest point of its rising phase. The possibility of latency variations due to spontaneous excitability changes was checked by a fixed surface recording electrode and comparison of the surface and depth records on a dual beam oscilloscope.

Direct commissural activation of CA1 neurons. When recorded from the various depths of the CA1 cortex the latency of the spike activated by a direct commissural volley showed a minimum in the basal part of the stratum radiatum (Fig. 4), increasing in both directions along a line, parallel to the apical dendritic shafts. Measurement of these latency variations (along 15 electrode tracks in 9 rabbit and 3 cat experiments) gave conduction velocities in the mentioned directions from 0.18 to 0.6 m/sec, in average 0.35 m/sec (Fig. 4 B and D). Note the small latency variation in the apical dendritic shaft layer, indicating either a high speed of propagation or a synchronous activation. The values were similar in cat and rabbit. No measurements were made in rat.

Sometimes the shortest spike latency was found in the cell body layer and increased with more superficial electrode locations. This pattern was obtained when the cortex was highly excitable, or when the stimulating electrode was moved from CA1 to CA2 or CA3 on the contralateral side. The speed of conduction of the spike was within the range given above.

Indirect activation of CA1 neurons by contralateral CA3 stimulation. Activation of the CA1 pyramids with the formation of a spike could also be produced by contralateral CA3 stimulation (ANDERSEN 1959). The form of the recorded potentials were similar to those described above, but sometimes the spike showed its shortest latency on the distal part of the apical dendrites. In these experiments the latency was longer than that of the spike evoked by a direct commissural volley. Fig. 5 A shows the potentials recorded from various depths of CA1 in response to contralateral CA3 stimulation. Fig. 5 C and D show that the spike had its shortest latency at a depth of 1.3 mm. This corresponds to the layer in which the Schaffer collaterals are found, suggesting that these fibres are responsible for the initiation of the spike. From the graph in Fig. 5 D the calculated conduction velocity along the course of the pyramidal apical dendrites is approximately 0.4 m/sec, a value comparable to that given above for the conduction of commissurally evoked spikes. The conduction in the most superficial part of the dendritic tree, *i. e.* towards the hippocampal fissure, was much slower, the velocity being 0.05—0.2 m/sec.

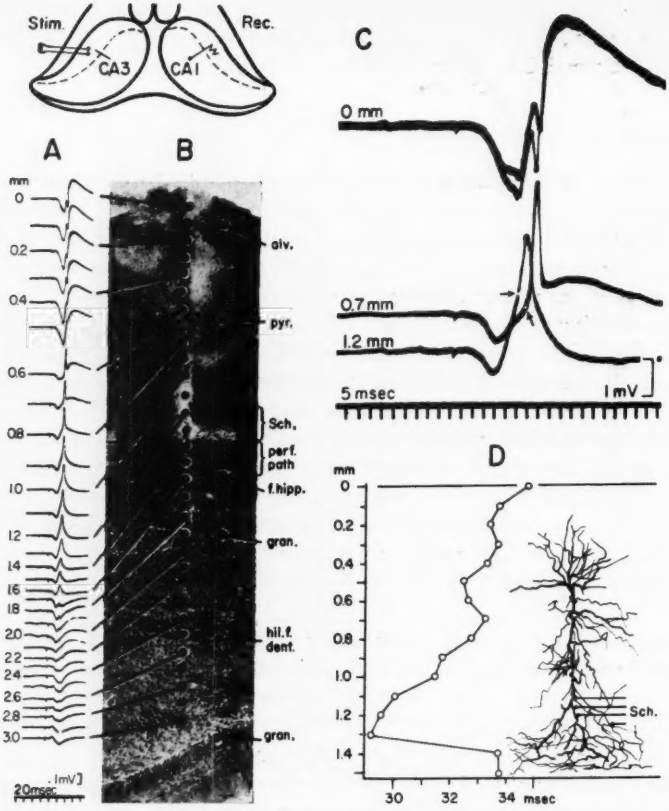

Fig. 5. Spike production in CA1 by contralateral CA3 stimulation. — *A.* Records obtained at different depths as indicated in *B.* — *C.* Records obtained 0, 0.7 and 1.2 mm below the ventricular surface are arranged to facilitate comparison of the spike latency at the three depths. The arrows in the two lowest records indicate the start of the spike. The two upper records are the surface records obtained simultaneously with the two lower. Slight spontaneous latency variation is seen. — *D.* Graph illustrating the variation of the spike latency at different depths in the CA1 cortex. A CA1 pyramidal cell is drawn to scale and at the correct level for the purpose of comparison.

C. Excitability cycle of the activated CA1 commissural potential

Paired stimuli. The results reported in this section are based upon 43 experiments in 16 rabbits and 8 cats. When not otherwise stated, the intensity of the conditioning and the test stimuli were identical. The use of the terms absolute and relative refractory period means that maximal or supramaximal stimulation has been employed. Fig. 6 illustrates the excitability cycle of the

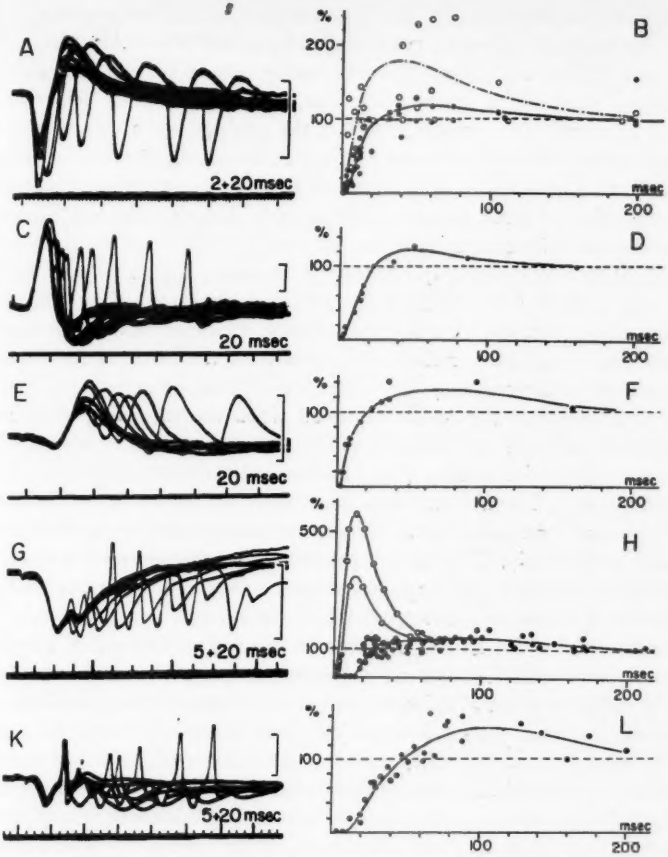

Fig. 6. Excitability cycle of the various components of the CA1 commissural response as tested by paired stimuli. Explanation in the text. Voltage calibration 1 mV.

three main components of the CA1 commissural potential, the positive and negative waves and the spike, as tested by paired stimuli. In the left part of the figure single experiments are shown, while the graphs in the right half represent data obtained from several experiments. The first pair of figures, *A* and *B*, show the results when both the conditioning and the test surface responses were without a spike. *A* shows the photographical superimposition of 12 sweeps with different stimulus intervals. *B* is a graph where the filled circles mark the amplitude of the positive wave of the test response in per cent of the corresponding component of the conditioning response. The open circles represent the corresponding values for the negative wave. The absolute refrac-

tory period was the same for the two components and measured about 2.5 msec. The relative refractory period of the positive wave was about 20—25 msec, and that of the negative wave shorter, about 10 msec. Even though the positive wave of the test response was partly depressed at delays between 2.5 and 25 msec, the remaining part of the positive wave was summated to the conditioning response as shown in Fig. 6 A. Following the relative refractory period, there was a long-lasting (100—300 msec) period with increased test responses affecting both potential components. Usually, the percentage amplitude increase of the negative wave was greater than that of the positive wave.

C and *D* in Fig. 6 similarly illustrate the results obtained in an experiment where the surface CA1 commissural response consisted of an almost pure negative wave (increased cortical excitability, 12 superimposed sweeps). The absolute and the relative refractory period were the same as for the surface positive wave of the ordinary CA1 commissural response.

E and *F* illustrate the results obtained when the recording electrode was placed in the layer of the apical dendritic shafts of the CA1 neurons, 1.0 mm below the ventricular surface (the commissural radiatum response, 12 superimposed sweeps). The absolute and the relative refractory periods of the deep negative wave were the same as for the surface positive wave, 2.5 msec and 25 msec respectively. The deep negative wave also showed a long-lasting period with increased test responses from 25 to about 200 msec delay. The summation of the two responses, even at the smallest delays, is shown in *E*.

G and *H* show the results obtained by paired stimuli when a spike was present in the conditioning surface response (12 superimposed sweeps). Two entirely different types of spike behaviour were obtained — one is illustrated by the superimposed records in *G* and the open circles in *H* (test spike amplitude in per cent of the conditioning spike magnitude) — the other is marked by the filled circles in *H*. Results as illustrated in *G* was seen when the spike of the conditioning response was small. With such conditions the test spike showed a marked amplitude increase with a maximum at 5—12 msec delay. This delay corresponds approximately to the stimulus separation giving the maximal summated amplitude of the surface positive or the deep negative waves of the conditioning and the test responses (*cf.* Fig. 7 C). As the conditioning stimulus was submaximal the experiment did not give information about the absolute refractory period of the spike, but the spike was observed at delays down to the absolute refractory period of both the surface positive wave (*B*) and the deep negative wave (*F*), about 2.5 msec. However, if the stimulus intensity was increased so as to produce a maximal spike in the conditioning response, the test spike showed an absolute refractory period of about 15 msec (filled circles in *H*). The relative refractory period of the spike was 20—40 msec. This period was followed by a period of 100—200 msec with increased test spike amplitudes, much like the case for the surface positive and the deep negative waves.

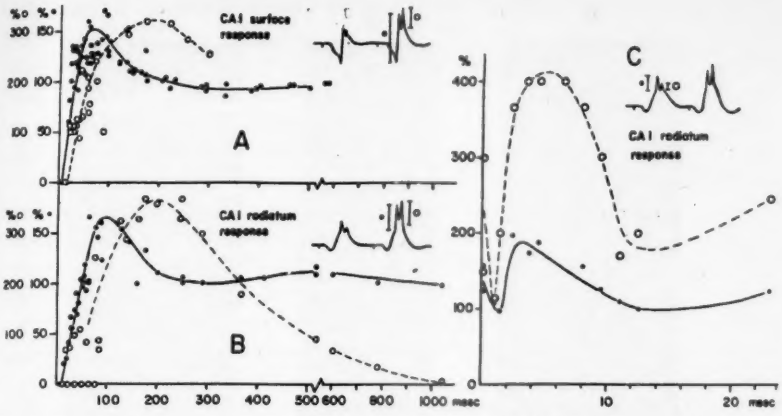

Fig. 7. Excitability cycle of the CA1 commissural potential when two spikes were present. Explanation in the text.

K and *L* show the results when a maximal spike was present in the conditioning response, as recorded from the layer of the apical dendrites, 1.2 mm below the ventricular surface. In this experiment the long absolute and relative refractory period of the spike is clearly demonstrated as well as the subsequent long-lasting facilitation.

When the single shock response of CA1 in response to contralateral CA3 stimulation showed the same latency as the usual CA1 commissural response, the excitability cycle — as tested by paired shocks — was as described for the latter potential. However, when the latency of the CA1 potential produced by contralateral CA3 stimulation was longer (spike latency 24—35 msec in rabbits) both the absolute and the relative refractory period of the positive wave and the spike was longer than that of the CA1 commissural potential.

When two spikes were present in the CA1 commissural response, they possessed different excitability cycles. Fig. 7 A shows a graph where the amplitudes of the two test spikes in per cent of the amplitudes of the respective single shock spikes were plotted against the stimulus interval (see inset). The absolute refractory period of the second spike (open circles) was about twice that of the first spike (dots). The relative refractory period and the culmination time of the subsequent facilitation of the second spike was longer than the corresponding features of the first spike, again approximately doubling the figures. Fig. 7 B illustrates the results obtained with the same technique, but recorded from the stratum radiatum, 1.0 mm below the ventricular surface. In *A* and *B* note the relatively greater facilitation of the second spike as compared with the first (different ordinate scales). The spread of observations on the second spike is partly due to difficulties in measurement as it often merges into the

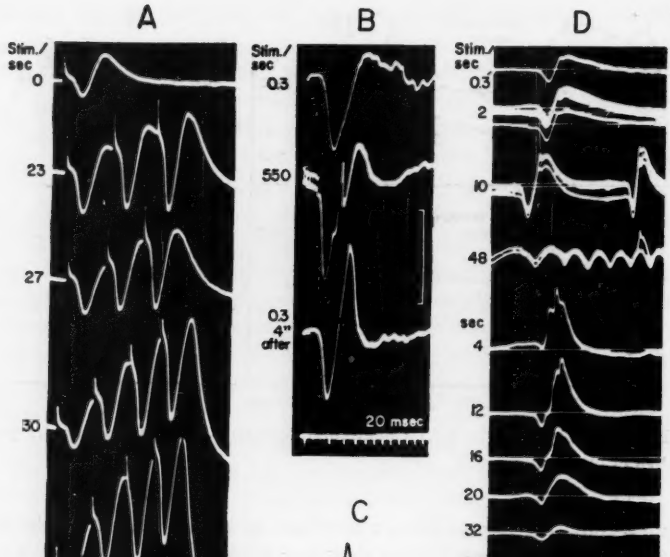

Fig. 8. Effect of repetitive stimulation on CA1 commissural response. — *A*. Surface response as affected by increasing stimulation frequency. Upper record single shock response. — *B*. Effect of short burst of tetanic stimulation. Upper record before, lower record 4 sec after cessation of the tetanic stimulation. — *C*. Upper and lower records in *B* superimposed. — *D*. Effect of repetitive stimulation of increasing frequency on CA1 commissural radiatum response. Following the stimulation first post-tetanic potentiation, then depression is seen. Voltage calibration 1 mV.

first spike. This is especially evident for the smallest amplitudes of the second spike. *C* shows the increase of both spikes of the conditioning shock at stimulus intervals of 2—12 msec. These figures correspond to the greatest amplitude of the summated deep negative wave. The increase at zero delay compared with 1—1.5 msec delay indicates that the stimulus intensity in the experiment illustrated in *C* was submaximal.

Repetitive stimulation (2—35/sec) produced recruitment of both the positive and the negative component of the CA1 commissural surface response (Fig.

8 A). Similar observations were obtained from all three species (112 experiments in 12 cats, 24 rabbits and 4 rats). The most efficient frequency was usually about 30/sec. At higher frequencies the facilitation subsided. The component to be augmented depended on the form of the single shock response. Low excitability of the hippocampal cortex with a well developed surface positive phase was associated with greater recruitment of this part than of the negative wave (Fig. 8 A). Conversely, repetitive stimulation during periods of increased irritability resulted in a prominent recruitment in the surface negative phase. The facilitated responses summated. The summated negative waves were often recorded as a long-lasting wave upon which the positive waves were superimposed, as seen in Fig. 8 A, 23—33/sec.

When spikes were absent in the single shock response one or more spikes could be elicited by repetitive stimulation at 3—40/sec, or by a short burst of very high frequency (Fig. 8 B). Long-lasting stimulation at frequencies above 40/sec depressed the later part of the potential including the spike (Fig. 8 A, 37/sec). However, the earliest part of the positive potential remained almost unchanged up to frequencies of 250/sec.

High-frequency stimulation (100—250/sec) in bursts every other second for 15—30 seconds often resulted in a long-lasting negative potential (200—300 msec) followed by a positive late potential. A similar, but smaller, effect may be seen in Fig. 8 A, 30 and 33/sec. The magnitude and the duration of the positive late potential increased with increasing stimulus frequency and duration.

A test response delivered at various intervals after the high-frequency stimulation showed an amplitude increase affecting all parts of the potential, especially the negative component (Fig. 8 B and C). As seen in Fig. 8 C the negative wave often showed a shorter latency after tetanic stimulation. The described post-repetitive effects lasted from some hundred milliseconds (including the positive late potential) to several minutes (4—6) after the cessation of the tetanic stimulation.

In records from the layer of the apical dendritic shafts a train of stimuli produced principally the same effects as reported for the surface response (Fig. 8 D). At frequencies from 2—35/sec all parts of the response increased, especially the negative wave and the spike. The recruiting rate was largely dependent on the prestimulatory amplitudes.

Stimulation at frequencies higher than 40/sec caused depression of the negative wave and the spike (Fig. 8 D at 48/sec). The negative wave broke through once bearing a spike on its summit. On cessation of the repetitive stimulation a long-lasting facilitation of the commissural radiatum response occurred (Fig. 8 D). The augmentation concerned the negative wave and the spike, and often new spikes were formed. The degree and the duration of this facilitation was proportional to the frequency and the duration of the repetitive stimulation.

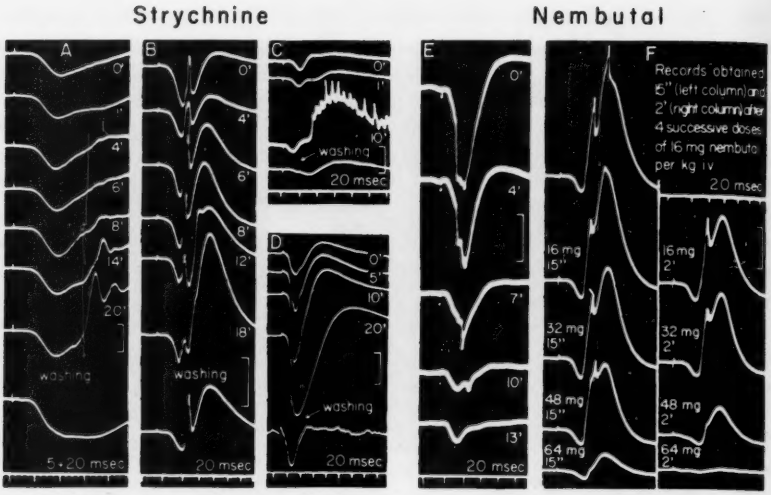

Fig. 9. Effect of local application of 1 per cent strychnine and 6 per cent sodium pentobarbital (nembutal) on CA1 commissural response. — *A.* Typical strychnine effect on surface response. — *B.* Effect of strychnine on fully developed surface potential. — *C.* Typical strychnine effect on deep response. — *D.* Unusual strychnine effect on surface response. — *E.* Effect of locally applied nembutal on surface response. — *F.* Effect of repeated doses of intravenous nembutal on deep response. Voltage calibration 1 mV.

The cortex was often unresponsive for several minutes subsequent to the facilitatory period (Fig. 8 D). Similar effects were observed during and after spontaneous and induced afterdischarges and will be discussed in more detail elsewhere (ANDERSEN 1960 c). After such depressions the gradual reappearance of the surface CA1 potential started with the development of the earliest, then of the later part of the surface positive component followed by the surface negative wave and finally by the evolution of the spike. This demonstrates the high resistance to fatigue of the surface positive potential, especially its earliest part, as compared with the other parts of the potential.

The reappearance of the commissural radiatum response is seen in Fig. 8 D. The early positive and the negative wave occurs before the spike. The last record shows a small afterdischarge, demonstrating the cortical hyperirritability which often follows the unresponsive period.

D. Effects of various drugs, anoxia and cooling on the CA1 commissural potential

Strychnine. Local application of 0.1—1.0 per cent strychnine sulfate solution to the recording point produced changes as illustrated in Fig. 9, all of which reversed on washing the cortex with warm Ringer's solution (23 experiments

in 6 cats, 15 rabbits and 2 rats). Four representative examples are shown, *A*, *B* and *C* being the most common. *A* illustrates the effect on the spike-free CA1 commissural potential. The surface positive wave was not significantly changed. However, the later part of the potential initially showed the development of a small positive wave which soon was reversed to negativity. On the summit of this new-formed wave, one and later two negative spikes occurred. *B* shows the effect on the fully developed CA1 commissural potential. Again, the first part of the response, including the positive wave and the spike, underwent only minor variations. However, the surface negative wave increased markedly in amplitude. Usually the duration as well was increased, as seen in Fig. 10 D and E. Riding on the rising phase or the summit of the enlarged surface negative wave one or more spikes appeared.

Fig. 9 C illustrates the effect on the commissural radiatum response. The early positive wave and the first part of the negative wave was not significantly augmented, but the later part of the negative wave was markedly prolonged and enhanced. On its top a series of spikes occurred. The fully developed evoked strychnine discharge started with small asynchronous spikes, becoming more and more synchronized and greatly increased in magnitude. This pattern was remarkable stable sweep to sweep.

The least common effect is shown in *D*. Both the amplitude and the duration of the surface positive wave increased, and a subsequent negative wave of long latency and duration was formed.

By use of stronger solutions (*e. g.* 5 per cent strychnine sulfate) the responses were gradually depressed. All parts of the potentials were affected and no period of increased responsiveness was noted.

For discussion of the strychnine effects see ANDERSEN (1960 a).

Sodium pentobarbital (*Nembutal* — "*Abbot*"). Local application of 6 per cent nembutal changed the CA1 commissural potential as demonstrated in Fig. 9 E and F. The surface response (E) consisted of the usual diphasic wave with three positive spikes superimposed on the positive phase. The effect of nembutal was to diminish the negative and the positive wave. After 7 min all but the first spike were abolished. The remaining spike decreased in amplitude while its latency increased. After 13 min only the earliest part of the surface positive wave remained. Prolonged nembutal application (30 min) had very little effect upon this part of the response.

The CA1 commissural radiatum response was also affected by the local surface application of nembutal, but the latency of the effect was variable and often only small potential changes were noted. However, nembutal given intravenously acted rapidly on the radiatum response. In Fig. 9 F the control record shows one spike on the rising limb and another spike on the summit of the deep negative wave. With increasing doses of nembutal the amplitude of the deep negative wave was decreased concomitant with the disappearance of the spikes.

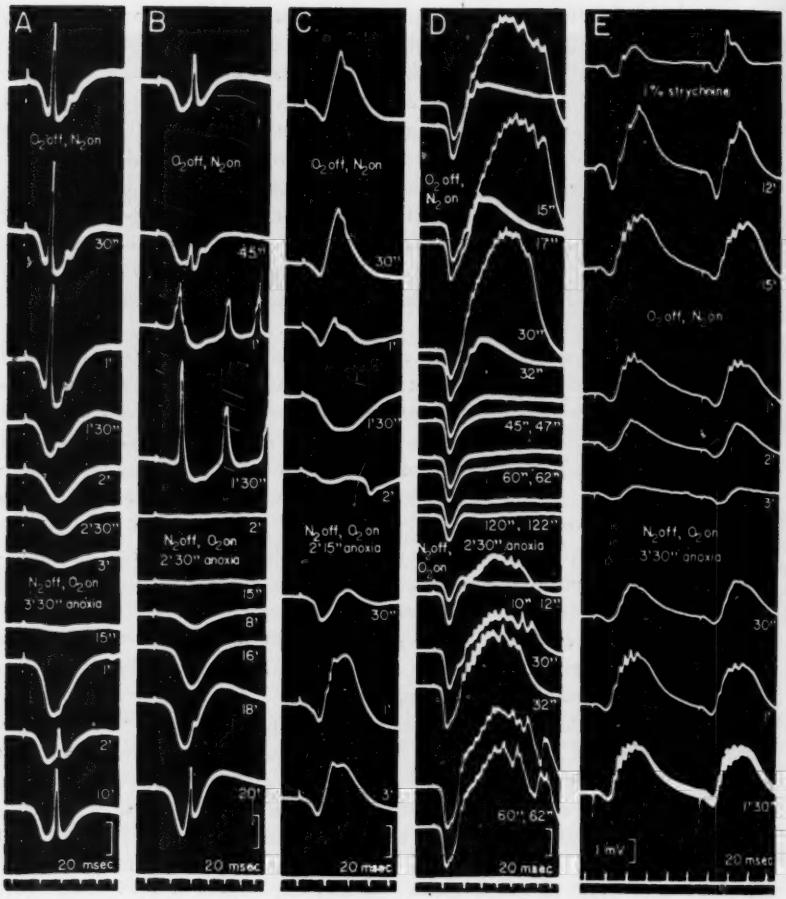

Fig. 10. Effect of anoxia alone and combined with strychnine on the CA1 commissural potential. — *A*. Effect of anoxia on the usual surface potential. — *B*. Afterdischarges induced by anoxia. Note the earlier disappearance of the potential, and the markedly increased restitution time as compared with *A*. — *C*. Effect of anoxia on the deep response. Post-anoxic hyperirritability is present. — *D*. Effect of anoxia on a lightly strychninized CA1 cortex. Following oxygen readministration the cortex shows signs of increased irritability. Further description in text. — *E*. Effect of anoxia on the CA1 commissural potential showing marked signs of strychnine intoxication. Similar susceptibility and restitution time as compared with *A*. Voltage calibration 1 mV.

The latent period before the full effect of the intravenously injected nembutal was relatively long as seen by the comparison of the responses obtained 2 min after the injection with those recorded only 15 sec afterwards.

Procaine (in a 2 per cent solution), applied to the ventricular surface of the hippocampus, almost simultaneously diminished all components of the commissural potentials. However, in some experiments the surface negative wave and the spike disappeared before the surface positive wave. The spike of the radiatum response was abolished concomitant with the diminishing of the negative wave.

Anoxia. Replacement of the animal's oxygen supply with nitrogen gave results as illustrated in Fig. 10. Observations on anoxia effects were made in 64 experiments in 12 cats and 22 rabbits. The CA1 commissural surface response lost its spike components after 1—1½ min (A), and the latest spikes were the first to disappear (note the increased latency of the first spike at 1½ min). Then followed the concomitant abolition of the negative wave and the major part of the positive component, leaving only the earliest part of this wave visible. The latter component sometimes disappeared simultaneously with the rest of the positive wave, but could resist anoxic conditions for 8 min at 35° C. Upon re-institution of the oxygen the potential was completely restored, usually in 1—2 min, unless the anoxia lasted for more than 4 min at a local hippocampal temperature of 35° C. At this temperature full restitution was seen after 4—5 min of anoxia, while cooling to 24° C increased the anoxic survival time of the potential to 8—10 min. The reappearance of the different potential components took place in the reverse order of their disappearance.

In about two thirds of the experiments a post-anoxic hyperirritability was noted. This condition was characterized by higher spike amplitudes and an increased number of spikes in the restitution period after the anoxia compared with the pre-anoxic potential. This increased excitability lasted from some seconds up to 3 min. In addition, in some experiments hyperirritability was also noted 20—60 sec after the start of the anoxia. It could be displayed by increased spike magnitudes as in *A*, or by the production of sustained electrical discharges as shown in *B*. The records marked 1′ and 1′30″ show that a train of discharges could be driven by the commissural volley. The usual rhythmic and continuous discharges evoked in this manner seldom lasted for more than one minute even if the oxygen supply was restored. When the anoxia was continued the cortex failed to respond to the commissural volley shortly after the cessation of the seizure discharge. In experiments showing anoxia-induced afterdischarges the restitution period following readmission of the oxygen was markedly increased compared to the restitution after an anoxia period of similar length which did not provoke afterdischarges (compare *A* and *B*).

The effect of anoxia upon the commissural radiatum response is seen in *C*. Shortly after the deprivation of the oxygen, the negative wave was sometimes augmented and the number of spikes riding on it increased. After 1 min the negative wave was diminished and below a certain magnitude of this potential

no spikes were seen. The negative wave was most often replaced by a flat record but sometimes, as in the experiment illustrated, a long-lasting positivity occurred. The negative wave ordinarily resisted anoxic conditions for 3—4 min at a hippocampal cortical temperature of 35° C, which is comparable to the corresponding properties of the major part of the surface positive wave. On oxygen readmission the potential components returned in the reverse manner. In the majority of the experiments (10 of 14) a post-anoxic hyperirritability was noted with an augmented amplitude of the negative wave and an increased number of spikes (C 1′).

The post-anoxic hyperirritability is clearly illustrated by the experiment shown in Fig. 10 D. In the slightly strychninized hippocampal cortex the CA1 surface response to a constant contralateral stimulation was alternating as in the first two records. This alternating sequence was observed for more than 15 min. In the rest of the figure pairs of records are mounted, each pair represents two successive sweeps with an interval of 2 sec. The anoxia abolished the late, prolonged negative wave with the superimposed spikes after 36″, while the initial positive wave was little changed by 2 min of oxygen lack. In the restitution period the records did not show such alternating forms as before the anoxia. The increased responsiveness of the hippocampal cortex in this experiment lasted for $2^1/_2$ min.

Fig. 10 E illustrates the combined effect of strychnine and anoxia on the CA1 commissural potential. The control record shows a typical facilitation of the CA1 surface response by a conditioning shock preceding the first one by 100 msec. Local application of 1 per cent strychnine sulfate produced a marked increase in the negative wave with the development of a series of superimposed spikes. However, the facilitation from the test response vanished. Under anoxic conditions the surface negative wave was diminished and the number of spikes decreased. After 3 min anoxia, however, still some of the negative wave was present. The restitution time following an anoxic period of $3^1/_2$ min was just as long as in the experiments without strychnine, and the post-anoxic record did not differ qualitatively from the control potential. The last record shows five superimposed sweeps to demonstrate the constancy of the potential.

Cooling. Local cooling of the hippocampal cortex (3 rabbit experiments) gave results similar to those obtained by general body cooling (4 rabbit experiments). As the temperature was lowered, the potential showed no significant change until the hippocampal surface temperature reached 30° C. Between this level and 24° C all parts of the surface potential were augmented and the number of spikes on the positive wave were increased. In addition, the latency and the duration of all components increased approximately in reverse proportion to the hippocampal surface temperature. It did not seem as if any part of the CA1 commissural potential was more susceptible to cooling than other parts. The potentials were obtained down to 18° C, where heart failure occurred, as judged from the electrocardiogram.

Discussion

Pattern of response. No significant differences were observed between the CA1 commissural potentials recorded from cat, rabbit and rat. Since the greatest number of experiments were performed in rabbits, the results discussed are as obtained from this animal when not otherwise stated.

When recorded from the ventricular surface of the hippocampus the form of the CA1 commissural potential is similar to the records presented by CRAGG and HAMLYN (1957) who also stimulated the contralateral hippocampus. The same form has been observed from the dorsal surface of the hippocampus (presumably the CA1) following stimulation of the entorhinal area (RENSHAW, FORBES and MORISON 1940, ANDERSEN 1958), of the dorsal fornix (EULER *et al.* 1958), and of the septal nuclei (KAADA, ANDERSEN and BRULAND 1959).

The form of the CA1 commissural potential is also comparable to the usual neocortical and cerebellar cortical responses to an afferent volley (BREMER 1958).

In the present investigation, recording by a penetrating electrode showed that the deep negative wave corresponded to the major, later part of the surface positive wave. A similar polarity reversal of the initial wave of the response to a synchronous afferent volley has been noted by several authors investigating both the neocortex and the cerebellar cortex (for references see BREMER 1958) and the hippocampus. In the latter cortex polarity reversal of the surface positive wave has been noted following entorhinal stimulation (RENSHAW *et al.* 1940, ANDERSEN 1958), stimulation of the contralateral hippocampus (CRAGG and HAMLYN 1957), and of the dorsal fornix (EULER *et al.* 1958).

The deep negative wave recorded from the neocortex has usually been interpreted as the sign of the depolarization of the soma and the basal dendrites. The results obtained by commissural activation of the hippocampus indicate that the surface positive wave of the CA1 is due to the depolarization of a more restricted portion of the dendritic tree, the apical dendritic shaft. Thus, the same form of the surface potentials may reflect different localization of the activated synapses in relation to the recording electrode. Further, the observations from the inverted hippocampal cortex suggest that the form of the surface potential is largely dependent upon the magnitude of the depolarized area and its distance from the recording electrode. Whether the recording electrode is placed at the surface of the cortex or upon the subjacent white matter seems to be of less importance.

The commissural radiatum response. The negative wave of the CA1 stratum radiatum, *i. e.* the layer of the apical dendritic shafts of the pyramidal cells, in response to a synchronous commissural volley bears some resemblance to an excitatory postsynaptic potential, EPSP (ECCLES 1957). The properties which the radiatum negative wave shares with an EPSP may be summarized as follows: (i) similar form with a sharply rising limb and a slower, nearly

exponential decay; (ii) constant duration, independent of the stimulus strength; (iii) negative polarity when recorded extracellularly; (iv) ability to summate; (v) short absolute refractory period; (vi) moderate susceptibility to nembutal and anoxia; (vii) high resistance to strychnine; (viii) apparently causal relation to the spike production.

However, the duration of the radiatum negative wave and its rising phase are longer than the corresponding features of the motoneuron EPSP (Brock, Coombs and Eccles 1952, Eccles *et al.* 1954; Eccles 1957). On the other hand, EPSPs of comparable duration and rising time have been recorded intracellularly from Betz' cells of the cat motor cortex (Phillips 1956, 1958) and from sympathetic ganglion cells of the rabbit (R. M. Eccles 1955). The EPSP of the Renshaw cells in the spinal cord normally have a duration of 50 msec but may be as long as 3 sec (Eccles 1957 p. 84).

The long duration and rising time of the radiatum negative wave may thus be due to the special properties of the activated neurons. Further, uneven slowing of the impulses in different terminal unmyelinated commissural fibres would give an increased rising time and an extended duration to the radiatum negative wave. Such an explanation has been proposed for the delayed appearance of the second and third spike of the primary response of the visual cortex (Bremer and Stoupel 1956, 1957).

In conclusion, the presented experimental data suggest that the negative wave recorded from the CA1 apical dendritic layer, in response to a commissural volley, is due to the summation of nearly simultaneously produced EPSPs in the pyramidal apical dendrites.

It cannot be excluded that other nerve cell structures may be responsible for part of this postsynaptic potential. However, in the region where the negative wave was recorded with the greatest amplitude and steepest rising limb, the apical dendritic shafts are the dominating elements, contributing by far the greatest total area of excitable neuronal membranes. Some part of the deep negative wave might be due to a prolonged depolarization of the terminal afferent fibres (Adrian 1941, Li, Cullen and Jasper 1956). However, it is unlikely that these structures would have as small a resistance to anoxia and nembutal as the postsynaptic structures. Furthermore, the electrical indications for the termination of an impulse in a volume conductor is dominated by the initial positivity (Lorente de Nó 1947, Lloyd 1951). For this reason the earliest part of the surface positive wave, which retains its polarity through the entire depth of the CA1 cortex, is considered to represent the impulse conduction in the terminal commissural fibres. This is consistent with the finding that this potential component has a high resistance to high-frequency stimulation, anoxia and nembutal.

The short absolute refractory period of the surface positive wave (*i. e.* in the vicinity of the commissural fibres, Andersen 1960 b) and the high resistance of parts of the CA1 commissural response to high-frequency stimulation,

anoxia and nembutal suggest that the major part of this potential is due to the activation of CA1 neurons by a direct commissural volley. As reported earlier this assumption formed the basis for the determination of the distribution of this potential (ANDERSEN 1959).

The observations on the latencies of the surface positive wave and the spike reported in this work indicate that the CA1 neurons may be activated in three ways from the contralateral hippocampus. These are (i) direct commissural activation by way of the apical dendritic shaft, (ii) direct activation from the contralateral field CA3, acting on the cell body or the basal dendrites, (iii) indirect activation from the contralateral field CA3 by way of the ipsilateral CA3 neurons and their Schaffer collaterals synapsing with distal parts of the CA1 apical dendrites. From known anatomical data the CA1 neurons may in addition be activated by impulses traversing the following route: commissural fibres to the granule cells of the dentate area — mossy fibres to the CA3 and CA2 neurons — Schaffer collaterals to the CA1 apical dendrites. The present work gives no information regarding this last impulse way.

The interpretation of the negative wave recorded in the stratum radiatum, as a sign of postsynaptic activity elicited in the apical dendrites of this region, receives strong support from anatomical studies. In normal material CAJAL (1911 p. 779) and LORENTE DE NÓ (1934) traced commissural afferent fibres to the stratum radiatum of CA1. Experimental histological studies have confirmed these observations. Thus, following contralateral destruction of the hippocampal formation BLACKSTAD (1956) observed terminal degeneration in two laminar zones of the CA1 field, both sharply delimited from the neighbouring structures. Heavy terminal degeneration was found in the stratum oriens, *i. e.* the layer of the basal dendrites, and in the stratum radiatum, the layer of the apical dendritic shafts. The degeneration was described as "massive, very finely granulated, and of about equal intensity at all laminar levels" (p. 489).

The results obtained by BLACKSTAD (1956) are at variance with those of CRAGG and HAMLYN (1957) who were not able to find terminal degeneration in CA1 following severance of the contralateral fimbria. As described, the findings reported in the present work corroborate the results of BLACKSTAD (1956).

From the present physiological data it seems that the terminal commissural fibres ending in the stratum radiatum more readily initiate postsynaptic activity than those ending in the stratum oriens. Three possible explanations can be mentioned. First, the threshold for initiating postsynaptic discharge may be lower in the apical dendritic shafts than in the basal dendrites. This does not seem unlikely, for the basal dendrites are much thinner than the apical dendritic shafts. Secondly the ipsilateral entorhinal afferents through the perforant path (CAJAL 1911, LORENTE DE NÓ 1934, RENSHAW *et al.* 1940, BLACKSTAD 1958) possibly exert an excitatory bombardment upon the terminal

branches of the CA1 apical dendritic tree. This would tend to diminish the threshold in the neighbouring part of the apical dendrites. Apart from a small contingent of fibres from the alvear path (LORENTE DE NÓ 1934) no similar afferent inflow is reported for the CA1 basal dendrites. The third explanation is that the fibres ending in the stratum oriens take their origin from contralateral fields other than CA1, presumably CA3 and CA4. This is indicated by the greater ease by which a purely surface negative wave is evoked by contralateral CA3 stimulation as compared with stimulation of CA1 (ANDERSEN 1959).

The part of the apical dendrites which serve as the generation region for the deep negative wave (EPSP) varied in the different experiments according to the responsiveness of the cortex. High excitability of the cortex was associated with commissural potentials exhibiting a prominent surface negative wave of short latency and great amplitude, and sometimes the surface record was even purely negative. This suggests that the EPSP, in periods of increased excitability, is generated in structures near the ventricular surface of the hippocampus. Therefore, the heightened excitability seems to be correlated with an increased synaptically depolarized area on the pyramidal neurons. The underlying mechanism is not revealed by the experiments in the present investigation.

The surface negative wave. The interpretation of the mechanism responsible for the production of the surface negative wave offers great difficulties. For reasons to be described below, it is proposed that this wave is composed of two parts. Since the threshold and the excitability cycle of the surface positive and the surface negative components, are the same, it may be suggested that the underlying mechanism of production for these two potential fractions is the same. This assumption is further strengthened by the observation of a gradual and reversible change of a potential with a prominent surface positive wave into a potential with a dominating surface negative wave (Fig. 1 C). Therefore, at least a part of the surface negative component may be considered as due to the electrotonic spread of the deep negative wave towards the basal parts of the excited neurons. This assumption is in agreement with the interpretation of the surface negative wave of the primary neocortical potentials (CHANG and KAADA 1950, BISHOP and CLARE 1952, BREMER 1952, TASAKI, POLLEY and ORREGO 1954, LI *et al.* 1956), and of the corresponding wave of the cerebrocerebellar potential (JANSEN 1957). However, the CA1 surface negative wave did not have all properties in common with the surface positive wave: (i) the former was only recorded in the basal part of the CA1 cortex; (ii) compared with the surface positive potential the surface negative wave was easier facilitated by a conditioning shock or by low frequency repetitive stimulation and earlier depressed by high-frequency stimulation; (iii) the surface negative wave was more susceptible to nembutal and anoxia, and (iv) it was more readily enhanced by strychnine. This suggests that the surface negative wave of the CA1 commissural potential is composed of two parts. The one having

properties like the surface positive wave may be due to the mechanism indicated above, while the other part may be produced by the activity of cells lying near the alveus, secondarily activated by the commissural volley. The latter part would then be the component displaying the great facilitation by low frequency repetitive stimulation, depression at high frequencies, the greater susceptibility to anoxia and nembutal, and the great amplitude increase following strychnine treatment. This assumption would represent a compromise between the opinion expressed in the preceding chapter concerning the surface negative wave as a spread of the deep negative wave along the dendrites towards the surface of the cortex and the assumption of EULER and RICCI (1958). These authors ascribed the surface negative wave of the primary auditory potential to the secondary activation of superficially located neurons.

Mechanism and site of initiation of the spike in the stratum radiatum. When the negative wave recorded in the apical dendritic layer was increased above a certain threshold, one or more spikes occurred on its summit or on its rising phase. In this layer the spikes were always recorded on a negative wave. Augmentation of the negative wave was associated with an increased number of spikes. Therefore, it seems that the negative wave has a causal relationship to the spike initiation.

Regarding the possible propagation of the observed spikes, the measurements of their latencies in the different cortical layers of CA1 are of crucial importance. The spike recorded in the stratum radiatum was always of negative polarity throughout the layer. The latency of the spike was measured to the point where the rising limb of the spike emerged from the negative wave. Of great importance is the fact that the variation of the start latency went parallel with the variation in crest time, and that the duration of the spike remained much the same at different laminar levels of the cortex. Therefore, as seen in Fig. 5 C, the whole spike shifted its latency, and not only varied the starting point of the spike. Any spontaneous shift of the latency of the spike would spoil the experiment. This was controlled by a recording electrode at the ventricular surface just above the penetrating electrode.

If the latency variation is accepted as a sign of propagation of the spike, the most probable structures along which the propagation might occur are the apical dendritic shafts. There can hardly be any question of the postsynaptic nature of the spike, and on the basis of its amplitude and its short duration, indicating a high degree of synchrony, the possibility of fibre activity being responsible for the spike seems small. Further, no histological substrate seems to be available for the propagation of an action potential of that order of magnitude in a basal direction. For these reasons the spike is regarded as an action potential initiated in and propagated along the apical dendrites of the CA1 pyramidal cells.

From the measurements of the spike latency, the region of generation was

usually found on the apical dendritic shafts with propagation of the spike both in a superficial direction, *i. e.* towards the end-ramifications of the apical dendrites, and in a basal direction, towards the cell bodies and further along the basal dendrites. The spike was conducted along the apical dendrite with a velocity of 0.35 m/sec (0.18—0.6 m/sec). This speed is in agreement with the value of 0.4 m/sec obtained by CRAGG and HAMLYN (1955) on direct intracortical stimulation of various afferents to hippocampal apical dendrites. As the spike recorded from the hippocampal cortex clearly differs from the "dendritic potential" observed by CHANG (1951) in the neocortex no correlation with the reported propagation velocity of that potential can be made.

Another, though less probable, explanation of the variation of the spike latency is that the successively synaptic depolarization of parts of the apical dendrite lying further away from the initial depolarization might help "pushing" forward an impulse which otherwise would be blocked.

The abrupt polarity reversal of the spike when the electrode invaded the layer of the terminal branches of the apical dendrites indicates that the propagation of the spike is often blocked at this point.

As a rule the amplitude of the spike increased near the cell body layer. The reason may be the movements of greater amounts of ions through the probably greater free surface of the neurons in the pyramidal layer compared with synapse-free areas of the apical dendritic shafts.

The possibility that the variations in the spike latency might be due to electrotonic spread of an inward current restricted to the pyramidal layer seems small. A conduction block between the soma and the dendrites would elicit a positive potential in the later, while spike was always purely negative in the entire stratum radiatum. Secondly, the latency variations because of electrotonic spread would probably be smaller than the observed values. Thirdly, the decrease of the spike amplitude with increasing distance from the soma seems too small to be explained by electrotonic spread only. Another objection to the electrotonic spread theory of the spike is the remarkably constant shape and duration of the spike as recorded in the different layers (Fig. 4 and 5).

From an extensive study of dendritic properties in the visual cortex CLARE and BISHOP (1955 a, b) have arrived at the conclusion that impulses only invade the apical dendrites electrotonically. Further, EULER *et al.* (1958) did not observe propagation of impulses down in the layer of the apical dendrites of CA2 in response to single shock stimulation of the dorsal fornix. However, such invasion was noted after repetitive stimulation. From these data it seems that the commissural activation of the CA1 apical dendrites may be more effective than certain other afferents in initiating a spike in this pyramidal cell structure. ARDUINI and POMPEIANO (1955) observed spontaneous spike production from the pyramidal layer, suggesting that different mechanisms of production may initiate spikes at different levels of the neuron. A direct comparison of the results of CLARE and BISHOP (1955 a, b)

with those of the present investigation can hardly be made as the latter authors observed the behaviour of the slow waves only.

The records obtained from the stratum oriens suggest that the action potential is propagated from the pyramidal layer towards the alveus with approximately the same velocity as in the apical dendritic layer. With small electrodes the spike was usually diphasic positive/negative in this layer indicating that the propagating spike passed the electrode (LLOYD 1951). Whether this propagation took place along the basal dendrites or along the pyramidal cell axons is difficult to decide because observations on the beginning of the myelin sheaths of these axons are lacking.

The production of action potentials elicited in the CA1 dendrites and conducted along them at a definite speed is in agreement with the findings of CRAGG and HAMLYN (1955). These authors observed small spikes riding on a long-lasting negative wave in response to near-by stimulation of various ipsilateral afferent fibres. The spikes showed increasing latency (when measured to the foot as well as to the crest of the spike) as the recording electrode was moved away from the layer of the stimulating electrode. This occurred both in the stratum radiatum and in the stratum oriens.

The speed of propagation of the spike parallel to the apical dendrites is small. The diameter of the apical dendritic shafts of the CA1 pyramids are from 2 to 8 μ as measured in Golgi sections[1]. The conduction velocity of C fibres (0.4—1.2 μ) is relatively higher, 0.7—2.3 m/sec (GASSER 1950) than the CA1 dendrite speed. However, if the calculation made for unmyelinated fibres in a great volume conductor (HODGKIN 1955) may be applied, fibres with diameters from 2 to 8 μ would conduct at 1.4 to 2.8 m/sec. Three morphologically observed factors would tend to bring the speed of propagation along the CA1 apical dendrites lower than this value. These are (i) the numerous branches, (ii) the narrowing of the shaft occurring at random, and (iii) the heavy covering of synapses (BLACKSTAD 1959). The branching and narrowing of the shaft would tend to delay the conduction in both somatopetal and somatofugal direction as the impulse in both instances would have to invade a greater membrane area. If the subsynaptic part of the cell membrane is electrically inexcitable as proposed by GRUNDFEST (1957), the great number of synapses would leave only part of the neuronal membrane free for the propagation of the impulses. These facts suggest that the observed value for conduction velocity is of the right order of magnitude for propagation along the unmyelinated apical dendritic shafts.

Properties of the CA1 apical dendritic membrane. On the basis of the experiments of CRAGG and HAMLYN (1955) and those reported in the present work, a hypothesis is ventured regarding the physiological properties of the CA1 apical dendritic membrane. One part of the membrane is thought to be responsible for the production of the EPSP. This is based upon the excitability

[1] The sections were kindly placed at disposal by Dr. L. E. WHITE JR.

properties of this potential component. The negative wave recorded from the layer of the apical dendritic shaft had an absolute refractory period that was similar to that of the afferent commissural fibres, it might be even shorter, or failing. Further, this wave had the ability of summation and was little influenced by a preceding spike discharge.

The other part of the membrane would then be responsible for the production and propagation of the spike. The spike had a definite absolute refractory period of about 15 msec, at appropriate intervals it was facilitated by a preceding deep negative wave and inhibited by a preceding spike. These excitability properties make it likely that the deep negative wave and the spike, both recorded from the layer of the apical dendritic shafts, are generated by different mechanisms.

The experiments with measurements of the spike latency suggest that the spike may be formed in and conducted along the apical dendritic shafts. This would infer that a part of the dendritic membrane is electrically excitable. However, PURPURA and GRUNDFEST (1956) have denied the propagation of impulses in neocortical apical dendrites and proposed that these dendrites are only chemically, and not electrically excitable. The observations made in the present investigation are not in accord with the theory of PURPURA and GRUNDFEST (1956). It seems unlikely that the properties of the neocortical apical dendrites should differ fundamentally from those of the hippocampal dendrites. PURPURA and GRUNDFEST (1958) used the local cortical response (ADRIAN 1936) as the test potential for apical dendritic properties, and studied its variations after the injection of various drugs. However, EULER and RICCI (1958), investigating the interaction between the local cortical response and the cortical response to medial geniculate stimulation concluded that the local cortical response did not engage the apical dendrites of those neurons deep in the cortex which gave rise to the surface positive wave of the thalamo-cortical response. As long as the exact mechanism and site of the initiation of the local cortical response is not known, it seems difficult to ascribe the depressive effect of any drug on this potential to a specific structure.

In favour of the electrical excitability of a part of the CA1 apical dendritic membrane is the observation of the low stimulus threshold for the CA1 commissural response when the stimulating electrode was placed in the layer of the apical dendritic shafts contralaterally (ANDERSEN 1959), the finding of spike propagation along the apical dendrites in response to near-by stimulation of afferent fibres (CRAGG and HAMLYN 1955), and the reported depolarization of the apical dendrites following repetitive stimulation of the dorsal fornix (EULER *et al.* 1958).

The possibility of spike initiation seems to differ for the different types of dendrites. Thus, the peripheral parts of the motoneuron dendrites do not seem to be able to generate and conduct action potentials (ECCLES 1957 p. 55). However, FATT (1957), on antidromical stimulation of the motoneuron,

ascribed a part of the recorded potential as due to the invasion of the impulse into the dendrites. Because of the great difference in morphology and synaptic arrangement between the dendrites of the motoneurons and those of the cortical pyramidal cells it is not unlikely that they possess different properties.

GRUNDFEST (1957) has proposed a general theory about electrical inexcitability of the subsynaptic part of the cell membrane. If this theory will apply to the CA1 apical dendrites, the initiation of spikes in the apical dendrites and the propagation along them would demand that a part of the CA1 apical dendritic membrane is free of synapses. Electronmicroscopy of these dendrites has shown this to be true (BLACKSTAD 1959). The CA1 apical dendrites are heavily covered by synaptic knobs, but inbetween there are numerous parts of the dendritic membrane covered by glia, and in addition, small naked portions of the dendrites (0.1—0.3 μ in diameter). These morphological data are consistent with the assumption of electrical excitability of a part of the dendritic membrane.

The deep negative wave recorded from the layer of the apical dendritic shafts was not observed to be influenced by a preceding spike, even at short delays. Since deep negative wave is supposed to be an EPSP it seems as if this potential is generated in a part of the cell membrane that is not made refractory by a preceding discharge of the cell. This observation suggests that the theory of GRUNDFEST (1957) on electrical inexcitability of synapses is valid also for the commissural synapses on the CA1 apical dendrites.

Summary

1. The potentials recorded from the hippocampal field CA1 in response to stimulation of the contralateral symmetrical point in cat, rabbit and rat have been analyzed.

2. The normal pattern of response is a surface positive/negative wave with one or more spikes, usually negative, on the positive wave.

3. Depth recording revealed that the initial part of the surface positive wave retained its polarity through the cortex, while the following, major part of this wave reversed its polarity in the pyramidal layer and was recorded as negative in the layer of the apical dendritic shafts.

4. The negative wave recorded from this layer had a steep rising limb and a slower, nearly exponential decay. It lasted 25—60 msec. Over a certain magnitude, one or more negative spikes occurred upon its summit. The absolute refractory period of the deep negative wave was 2.5 msec, the relative refractory period 25 msec. The deep negative wave was relatively resistant to repetitive stimulation, was little affected by strychnine, was blocked by anoxia after 2—4 minutes, and was sensitive to pentobarbital. The deep negative wave is considered as a summated excitatory postsynaptic potential elicited in the

apical dendritic shafts of the CA1 pyramidal cells by a direct commissural volley.

5. The surface negative wave was only recorded between the ventricular surface and the cell body layer. From experiments with paired and repetitive shocks, application of strychnine and nembutal and anoxia, it seems that the surface negative wave is partly due to the electrotonic spread of the deep negative wave towards the ventricular surface, and partly to the secondary activation of neuronal elements lying near ventricular surface.

6. The spike had different latency in various layers of CA1. Usually the latency was shortest in the region of the apical dendritic shafts, sometimes a little more superficially, in the latter case suggesting that the Schaffer collaterals were responsible for the activation. The conduction velocity parallel to the apical dendritic shafts was 0.35 m/sec (0.18—0.6 m/sec). The spike had an absolute refractory period of 15 msec, and a relative refractory period of 40—50 msec. It followed 25/sec tetanic stimulation for a short period, it was unchanged by strychnine, but was the potential component showing the greatest susceptibility to anoxia and nembutal. The spike is regarded as an action potential initiated in and propagated along the apical dendrites, and sometimes into the basal dendrites as well.

7. The CA1 apical dendritic membrane is postulated to be composed of two parts. The first is responsible for the production of the deep negative wave (major part of the surface positive wave), and the other is responsible for the initiation of the spike in the apical dendrite, and the propagation along it.

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Interhippocampal Impulses

III. Basal Dendritic Activation of CA3 Neurons

By

PER ANDERSEN

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Abstract

ANDERSEN, P. *Interhippocampal Impulses. III. Basal dendritic activation of CA3 neurons.* Acta physiol. scand. 1960. *48.* 209—230. — The commissural afferents of the hippocampal field CA3 terminate in the layer of the basal dendrites only (BLACKSTAD 1956), making selective basal dendritic activation possible. The response of CA3 in response to a symmetrical contralateral stimulation is a diphasic spike followed by a negative wave, sometimes with a superimposed spike. The diphasic spike represents interhippocampal fibre activity. The negative wave was recorded only from the CA3 basal dendritic layer. It is partly an excitatory postsynaptic potential produced in the basal dendrites by the commissural volley, partly a postsynaptic CA3 neuron discharge. The spike is a more synchronous discharge of the same neurons. The commissural volley elicits spikes more seldom in CA3 than in CA1, where apical dendritic activation is the most prominent, suggesting that a cell discharge is easier produced by apical than by basal dendritic activation. The susceptibility to anoxia is similar for the two fields. Strychnine normally produces great changes in CA1 potentials, but has only minor influence on the CA3 commissural response.

From the field CA3 of the hippocampus a characteristic potential may be recorded in response to stimulation of a symmetrically located point contralaterally. This is termed the CA3 commissural response (ANDERSEN 1959). The main components of this potential are a diphasic spike followed by a negative wave, which sometimes had a superimposed spike.

Potentials with identical form have been obtained by CRAGG and HAMLYN (1957) following stimulation of the contralateral fimbria. Roughly similar responses have also been reported by LIBERSON, ELLEN and SMITH (1958 a, b) on commissural activation, but these authors do not state from which field of the hippocampus the potentials have been recorded. The same potential form has been recorded from the CA3 following stimulation of the ipsilateral fimbria (RENSHAW, FORBES and MORISON 1940) and the ipsilateral dorsal fornix (GREEN and ADEY 1956, DUNLOP 1957).

In an experimental histological study BLACKSTAD (1956) observed a fundamentally different pattern of termination of the commissural afferents to the field CA3 as compared with those to CA1. The difference between these two fields is also clearly shown in cytoarchitectonical studies by CAJAL (1911) and LORENTE DE NÓ (1934). Recently FLEISCHHAUER and HORSTMANN (1957) discovered a histochemical difference between CA3 and CA2 on the one hand, and CA1 on the other. Finally, COGGESHALL and MACLEAN (1958) have reported selective destruction of the CA3 neurons after the injection of 3-acetylpyridine while the CA1 pyramids remained seemingly unchanged.

The present work is an analysis of the CA3 commissural response in order to determine whether the differing potential form reflects different modes of commissural activation of the CA3 pyramids compared with the CA1 neurons, or not. Not only does CA1 differ from CA3 with regard to morphology and synaptical arrangement, but the susceptibility to anoxemia is said to be greater for the Sommer's sector (presumably corresponding to CA1) than for the rest of the hippocampus (SPIELMEYER 1925). For these reasons experiments were performed so as to correlate the influence of anoxia, strychnine and repetitive stimulation upon the CA3 commissural potential with the effect of the same procedures on the CA1 commissural potential.

Evidence is presented which makes it probable that the commissural volley activates the CA3 neurons through their basal dendrites, in contrast to the predominant apical dendritic activation of the CA1 pyramidal cells.

Material and Methods

The results are based on experiments in 24 cats, 0.17 (3 weeks old) — 6.8 kg; 24 rabbits, 1.6—3.8 kg; and 6 rats, 0.27—0.40 kg. Detailed description of the methods are given in a previous paper (ANDERSEN 1959). Urethane-chloralose were used for anesthesia. Only insignificant differences in the form and properties of the potentials were observed in control experiments with immobilization by decamethonium and local infiltration with procaine. Bipolar stimulating electrodes (polar separation 100 μ)

and monopolar recording electrodes (tip diameters 3—20 μ) were employed. A conventional recording arrangement was used with a large reference electrode. The push pull amplifier mostly used had time constants of 10 and 70 msec. All experimental results were controlled with an other amplifier with time constant 1 sec or by d. c. recording. The responses were tested by paired stimuli; repetitive stimulation; locally applied strychnine, sodium pentobarbital and procaine; anoxia; and finally by local or general cooling to hippocampal cortical temperatures of 18 °C. The procedures were as reported previously (ANDERSEN 1960 a). Histological verification of the electrode sites was made in silver impregnated sections (ANDERSEN 1956).

Results

A. Pattern of response

The CA3 commissural potential recorded from the fimbrio-hippocampal border following stimulation of the contralateral symmetrical point consisted of a diphasic positive/negative spike followed by a negative wave, and then a positive wave with one or more negative spikes superimposed (Fig. 1 A and B). The late positive wave with the spikes were only seen when the cortex was in good condition. At the beginning of the negative wave a small negative crest was often apparent. Stronger stimulation could elicit a negative spike on the negative wave (Fig. 1 B). However, this was considerably more difficult to obtain in CA3 than in CA1.

The threshold for the initial diphasic spike and the negative wave was the same, while the negative spike riding on the negative wave and the late positive wave with the small spikes had successively higher thresholds (Fig. 1 B).

In an electrode track, as illustrated in Fig. 1 D, the diphasic spike was recorded from the surface to a depth of about 0.8 mm, approximately corresponding to the pyramidal layer (Fig. 1 C and D). The latency of the diphasic spike increased with increasing depth, giving an intracortical conduction velocity in rabbit of about 5 m/sec, *i. e.* approximately the same as in the fibres of the fimbria (ANDERSEN 1960 b).

The negative wave increased in amplitude to a depth of 0.2—0.4 mm. At about 0.8 mm below the ventricular surface, corresponding to the cell body layer, the polarity was reversed (Fig. 1 C and D). Deep to the pyramidal cell layer this wave, now positive, gradually increased, both in amplitude and duration. Sometimes a sudden, dramatic amplitude increase was recorded — at 1.6—2.2 mm from the surface. This corresponds to a point in the vicinity of the inner part of the CA3 pyramidal layer and the lower extremity of the granule cell layer of the dentate area.

The early small negative crest in front of the surface negative wave underwent a steady increase in amplitude as the electrode was pushed down, and had a maximum at about 1.6 mm depth. This potential component here appeared as a spike with a duration of about 2 msec. A sudden marked increase

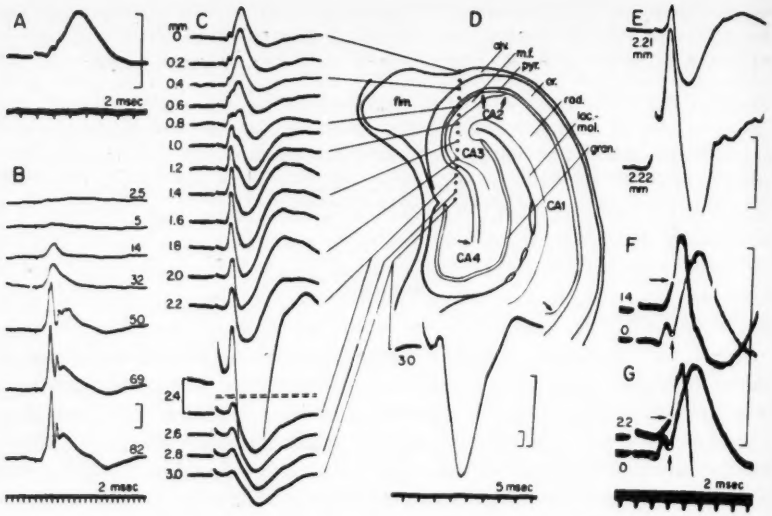

Fig. 1. CA3 commissural potential recorded with surface and penetrating electrode in rabbit. — *A.* Usual surface recorded CA3 commissural potential. — *B.* Effects of increasing stimulus strength, given in arbitrary units. Comparison of the lower two records shows that supramaximal stimulation may be obtained. — *C.* Records obtained at 0.2 mm intervals along the track indicated in *D*. The records below the double broken line are recorded with small amplification (small calibration line). — *E.* Comparison of potentials recorded only 10 μ apart at indicated depths. — *F* and *G* show the surface record superimposed on those obtained at 1.4 mm and 2.2 mm, respectively. The deep negative spike has a slightly shorter latency than the surface negative wave. All voltage calibrations 1 mV.

In all figures the recordings are monopolar and with negativity upwards.

Abbreviations: alv. — alveus, fim. — fimbria, gran. — granular layer, lac.-mol.-stratum lacunosum-moleculare, m. f. — mossy fibres, or. — stratum oriens, pyr. — pyramidal layer, rad. — stratum radiatum.

of both this deep spike and the deep positive wave could be noted when the electrode was moved as little as 10 μ, as shown in Fig. 1 E. However, the latency of the deep spike remained the same at different depths (Fig. 1 F and G).

B. Excitability properties of the CA3 commissural potential

Paired stimuli. Fig. 2 demonstrates the effect of a conditioning CA3 commissural response upon a subsequent similar test response. The diphasic spike of the test response was not influenced by the conditioning potential until the stimulus interval was 4—5 msec. At shorter delays the diphasic spike was diminished, and at 1.2—1.3 msec it was totally abolished in all three species (Fig. 2) (4 cat, 7 rabbit and 3 rat experiments).

The absolute refractory period of the negative wave was in all three species

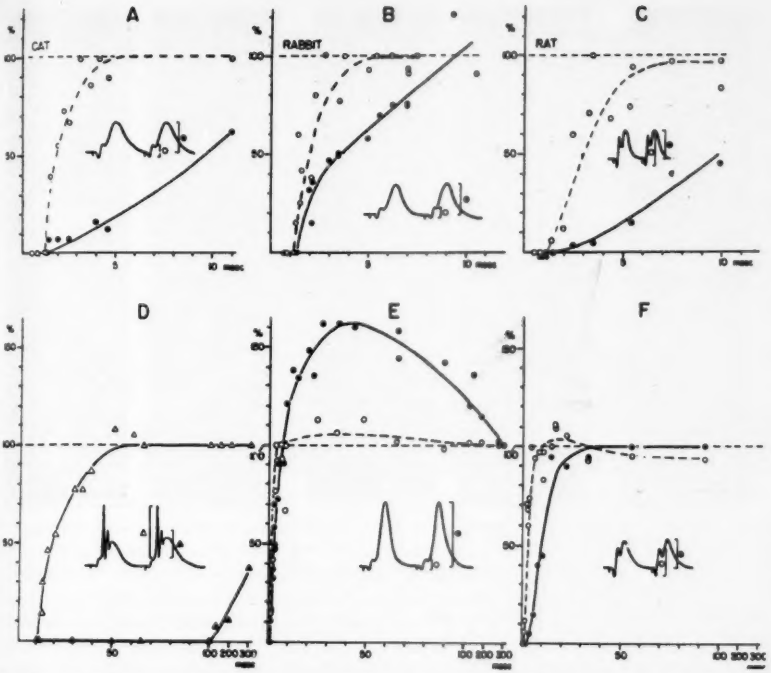

Fig. 2. Excitability cycle of CA3 commissural potential. — *A* shows the amplitude of the diphasic spike and the negative wave of the test response in per cent of the corresponding amplitudes of the conditioning response (see inset). — *B* and *C* illustrate the same in rabbit and rat, respectively. — *D*. The amplitudes of the first and second spike of the test response in per cent of the corresponding spikes of the conditioning response at various delays (cat). — *E*. Amplitude of the diphasic spike and the negative wave in per cent of the corresponding values in the conditioning response. Note that the negative wave here is of great amplitude (rabbit). — *F*. As in *E* but the negative wave is of low amplitude (rat). Effects as illustrated in *D*, *E* and *F* could be obtained from all three species.

1.3—1.5 msec while the relative refractory period measured 10—15 msec, *i. e.* longer than that of the diphasic spike (Fig. 2 A, B and C).

The three lower diagrams in Fig. 2 show the results obtained by paired stimuli at three different excitability levels. In *D* this level was high. The second spike is totally abolished even at 100 msec delay, while the first spike is depressed between 60 and 10 msec. *E* illustrates the results obtained in a period of moderately high excitability. The negative wave was then higher than usual in relation to the diphasic spike. Under such conditions the negative wave showed a marked facilitation between 8 and 2—300 msec. *F* illustrates an experiment in a period of low excitability. The negative wave was then poorly developed, and no facilitation occurred.

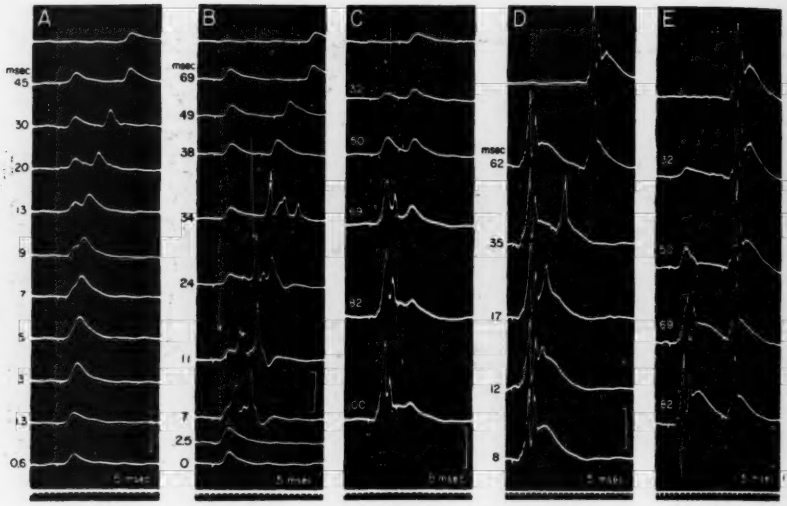

Fig. 3. Relation between the negative wave and the superimposed spikes. — *A*. Summation of the pure negative wave at delays from 30 to 3 msec. — *B*. Production of superimposed spikes on the negative wave at delays from 34 to 7 msec. Stimulation somewhat stronger than in *A*. — *C*. At a fixed delay of 22 msec the test negative wave is uninfluenced by the conditioning response even if the latter contains spikes. — *D*. Paired stimuli with supramaximal stimulation. Inhibition of the second spike at 62 msec, and of the first spike from 35 msec, being total at 8 msec. — *E*. Effect on a fully developed test response of increasing conditioning response at a fixed delay of 45 msec. The presence of a pure negative wave in the conditioning response facilitates both spikes in the test response. As soon as spikes occur in the conditioning response the spikes of the test response are inhibited, the second totally, the first by 40 per cent. All records from cat.

Fig. 3 illustrates the difference between the excitability cycle of the negative wave and that of the spike riding upon it, and the relation between these two potential components. *A* shows the small facilitation of the test response from 45 to 9 msec; the test response summated with the conditioning negative wave down to 3 msec. At 1.3 msec the response was as great as the conditioning response alone. In *B* the stimulus intensity of both shocks was somewhat increased. The elicitation of spikes on the test negative wave is seen from 34 to 7 msec, *i. e.* at the same delays as the test negative wave was facilitated in *A*. Because of the high excitability level in this experiment the relative height of the three spikes of the test response in *B* varied. If the first spike was high the third would be small and *vice versa*. In *C* the intershock delay was kept fixed at 22 msec. Increase of the conditioning response did not appreciably affect the size or form of the test negative wave. However, if the same experiment was made when the test response contained spikes, these would be depressed, but not until spikes occurred in the conditioning response (*E*).

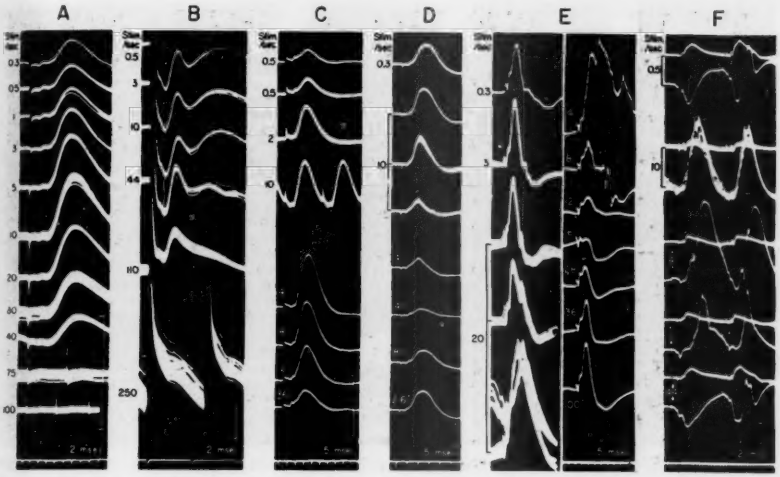

Fig. 4. Effect of repetitive stimulation on the CA3 commissural potential in rabbit. — Stimulating frequencies are indicated by black figures outside the columns; seconds after the cessation of tetanic stimulation are marked by white figures on the records. — *A.* Effect of increasing stimulating rates. — *B.* Different resistance of diphasic spike and the negative wave to high frequency stimulation. — *C.* Facilitation of the negative wave after tetanic stimulation. — *D.* Depression of the negative wave during and after tetanic stimulation. — *E.* Increase followed by depression of the negative wave and the late spikes after tetanic stimulation. — *F.* Different susceptibility of the CA3 commissural response (upper record in each pair) and the CA1 commissural potential (lower record) to repetitive stimulation. Left calibration line for CA3, right for CA1 potentials. Voltage calibration 1 mV.

D illustrates the effect on the spikes of decreasing delay between the paired shocks. The difference of the absolute refractory period of the first spike in *D* and that of the negative wave in *A* is clearly seen.

Repetitive stimulation. Various types of experimental results are illustrated in Fig. 4. *A* shows the usual effect of a short-lasting tetanic stimulation (less than 10 sec) on the CA3 commissural potential. At 3—30/sec the negative wave was augmented and started earlier than in the single shock response (every third second). Sometimes one or more spikes developed, but this occurred more seldom than in the field CA1 on commissural stimulation. At frequencies higher than 40/sec the negative wave was progressively depressed. The diphasic spike was still seen at 100/sec. The different susceptibility of the diphasic spike and the negative wave to tetanic stimulation is better seen in *B*. At 44/sec only a small part of the negative wave was seen, at 110/sec there was only a remnant of the negative wave whereas the diphasic spike was little affected. Even at 250/sec the diphasic spike was present.

Following high frequency stimulation (10—30/sec) for some seconds a period

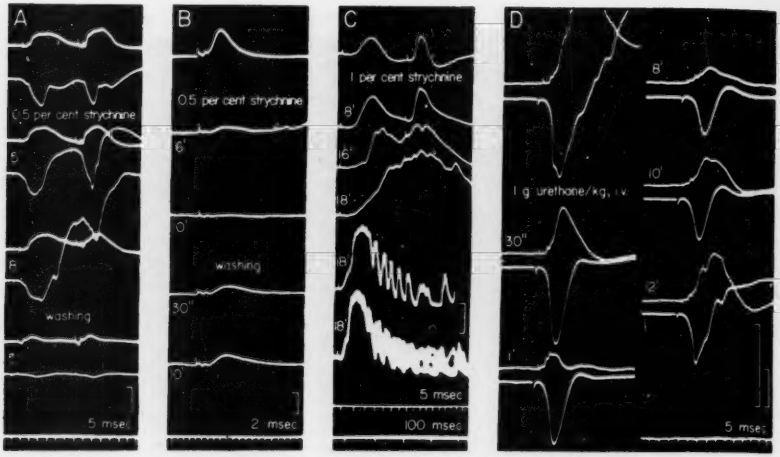

Fig. 5. Effect of strychnine and urethane on the CA3 commissural potential in rabbit. — Different susceptibility to strychnine of the CA3 commissural potential (upper record in each pair) and the CA1 commissural potential. — *B.* Depression of the negative wave of the CA3 commissural potential by low concentration of strychnine sulfate. — *C.* Occasional effect of strychnine on the CA3 commissural potential. The two last records obtained with slow sweep (calibration 100 msec). — *D.* Effect of a great intravenous dose of urethane (1.0 g/kg) on the CA3 and CA1 commissural potentials (upper and lower record in each pair, respectively). Left calibration line for CA3, right line for CA1 potentials. Voltage calibration 1 mV.

of increased excitability was often observed, as judged by increased CA3 commissural responses to a constant intensity single shock stimulus. The degree and the duration of this supernormality was increased with the frequency and the duration of the tetanic stimulation (provided that stimulation with more than 40/sec was not applied longer than about 10 sec). Fig. 4 *C* illustrates such an experiment where the post-tetanic increased responses were observed for about half a minute. The longest period of increased excitability was 6 min. The enhancement affected the negative wave, while the diphasic spike remained unchanged.

Howver, the CA3 commissural response often also underwent the opposite change following tetanic stimulation (Fig. 4 D). The records marked 10/sec were obtained 2, 6 and 10 sec after the start of the trigger stimulation. The depression again affected only the negative wave, leaving the diphasic spike unaltered. In this experiment the depression lasted about 3 min, but it could be observed for 30—40 min.

Sometimes an initial facilitation of the negative wave followed by a depression of the same was observed (Fig. 4 E). The records marked 20/sec were obtained 2, 10 and 22 sec after the start of the tetanic stimulation. The development of

several spikes on the negative wave is seen. Shortly after the cessation of the repetitive stimulation a long-lasting negative wave with several spikes appeared, but after 12 sec only a small part of the negative wave was left. The diphasic spike was not affected, neither in the supernormal nor in the subnormal phase. Only when long-lasting tetanic stimulation produced a profound unresponsiveness of the hippocampal cortex the diphasic spike was abolished. The restitution from this condition started with the reappearance of the diphasic spike, then the negative wave occurred and, finally, the superimposed spikes.

Short-lasting tetanic stimulation of 60—250/sec elicited a prolonged negative wave with several small sharp negative waves superimposed. With increasing stimulus intensity these small waves became smaller while the background negative wave increased in magnitude.

Post-tetanic potentiation of the commissural response could be more easily elicited from the field CA1 compared with CA3. Fig. 4 F shows that the CA3 commissural response (upper line) was depressed during and after the tetanic stimulation while the CA1 commissural response (lower line) showed a marked increase of its negative wave with the production of several superimposed spikes.

Tetanic stimulation often elicited selfsustained electrical afterdischarges. However, it was more difficult to produce such discharges in the field CA3 than in CA1.

C. The effects of various drugs, anoxia and cooling on the CA3 commissural potential

Strychnine. Local application of 0.1—1 per cent strychnine sulfate to the recording site had relatively small effects on the CA3 commissural potential in 6 cat, 12 rabbit and 2 rat experiments (Fig. 5 A, B, C). No influence on the early diphasic spike was observed. *A* is taken from an experiment where the strychnine was applied to both CA3 and CA1. The stimulating electrode was placed so as to elicit both the CA1 and the CA3 commissural potential. In CA1 0.1 per cent strychnine produced the typical effect with increase of the surface negative wave and the production of spikes on this wave. In CA3, only a small increase of the last part of the negative wave was seen after 8 min. *B* shows an example of the depressant effect of strychnine. Usually this depression occurred at concentrations above 1 per cent. The depression affected only the negative wave, leaving the diphasic spike unaltered. Sometimes the strychnine application produced an increase also of the CA3 commissural potential (Fig. 5 C). The effect consisted of an increase of the last part of the negative wave with the production of several sharp waves riding on the summit and the falling phase. The two last records in *C* are taken with slow sweep. The last one illustrates the high degree of synchrony of the late sharp waves, as demonstrated by 5 superimposed sweeps.

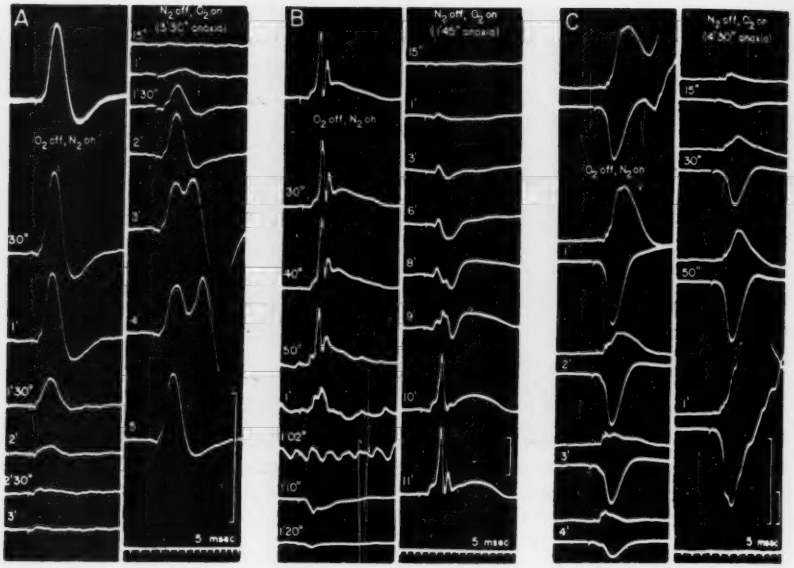

Fig. 6. Effect of anoxia on the CA3 commissural potential in rabbit. — *A.* Usual effect of anoxia affecting the negative wave, leaving the diphasic spike almost unchanged. Small increase after 30 sec of anoxia, great post-anoxic hyperirritability. — *B.* Anoxia-induced afterdischarges. Note the delayed restitution when compared with *A*, even if the anoxia only lasted half as long in *B* as in *A*. — *C.* Comparison of the susceptibility to anoxia of the CA3 and CA1 commissural potentials (upper and lower record in each pair, respectively). Left calibration line for CA3, right for CA1 potentials. Voltage calibration 1 mV.

Urethane. Rapid intravenous injection of 1.0 g urethane per kg body weight was followed by a diminished amplitude of the negative wave (Fig. 5 D). No effect was observed on the diphasic spike and the early notch of the negative wave. After 10 min the effect on the negative wave subsided, and full restitution was observed after 12—15 min. The effect on the CA1 commissural potential corresponded in time to that on the CA3 potential.

Procaine. Following application of a 2 per cent solution of procaine all parts of the response were diminished simultaneously. The diphasic spike was recorded for as long as 13 min after the application.

Sodium pentobarbital (*Nembutal-"Abbott"*). Local application of 6 per cent nembutal gave results qualitatively similar to those described for urethane, again showing different susceptibility of the diphasic spike and the negative wave.

Anoxia. The various components of the CA3 commissural potential were differentially influenced by anoxia (Fig. 6). Most susceptible were the late

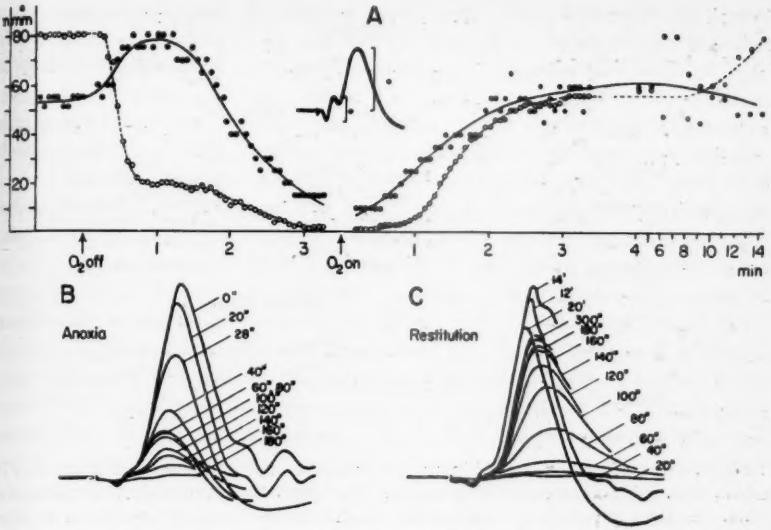

Fig. 7. Separation of the negative wave into two parts showing different resistance to anoxia. Rabbit. — *A*. Plotting of the amplitudes of the diphasic spike and the negative wave against duration of anoxia and time after oxygen re-admission (see inset). — *B*. Superimposed drawings of records obtained at indicated durations of anoxia. The culmination time of the negative wave decreases to 80 seconds of anoxia, thereafter it increases. — *C*. Similar drawing of the records obtained in the restitution period. The culmination time of the negative wave is gradually decrease from an initial great value. Note the post-anoxic hyperirritability with spike formation on the negative wave.

spikes, then the negative spike riding on the negative wave disappeared. After an anoxic period of 50—60 sec the negative wave was diminished (Fig. 6 A). The diphasic spike and the early notch of the negative wave were the most resistant (Fig. 6 A and C). In a number of experiments the diphasic spike was unaltered after 10 min of anoxia. In three long-lasting anoxia experiments the diphasic spike lasted for 40, 60 and 100 min. This was most probably due to direct oxygen uptake of the fibres from the air-saturated paraffin pool (*cf.* FRANKENHAEUSER 1949).

The time lag between the diphasic spike and the start of the negative wave increased during anoxia without any appreciable change in the latency of the diphasic spike.

The reappearance of the different parts of the CA1 commissural response took place in the reverse order to their abolition (Fig. 6).

In about one half of the anoxia experiments (14 out of 23) there was a post-anoxic hyperirritability indicated by a higher and longer lasting negative

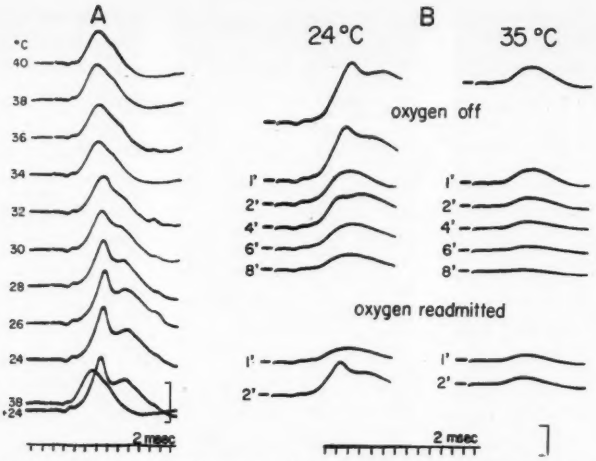

Fig. 8. Effect of cooling of the hippocampal cortex on the CA3 commissural potential in rabbit. — *A*. Effect of decreasing temperatures. Note the development of a spike on the negative wave. The last two records are superimposed to illustrate the increased latency and duration of all parts of the potential. The amplitude of the negative wave was approximately the same at 24 and 38° C. — *B*. Effect of 8 min of anoxia at indicated hippocampal cortical temperatures. The potentials obtained at 35° C are abolished earlier and show a poorer recovery compared with those recorded from the same animal at 24° C. Voltage calibration 1 mV.

wave with an increased number and amplitude of the superimposed spikes compared with the pre-anoxic response (Fig. 6 A, 3' and 4').

In some experiments the anoxia induced an electrical afterdischarge (Fig. 6 B). This was only observed when the cortex showed signs of high excitability. If the anoxia was maintained the afterdischarge subsided, leaving the preparation in a state of profound unresponsiveness. The occurrence of an afterdischarge within an anoxic period greatly delayed the restitution on readmission of oxygen to the animal, even if the anoxic period was short (compare *A* and *B*).

When the CA1 and CA3 commissural potentials were recorded simultaneously, their susceptibility to anoxia seemed similar (Fig. 6 C). The only slight difference which could be observed was that the surface positive wave of the CA1 commissural potential was abolished a little later than the negative wave of the CA3 potential (Fig. 6 C, 3' and 4' anoxia). The restitution followed a similar time course in the two fields, as did the post-anoxic hyperirritability. Both potential types usually resisted anoxia for 4—5 min at a local hippocampal temperature of 35° C.

Fig. 7 illustrates an experiment where the temporal sequence of the decrease

of the diphasic spike and the negative wave under anoxia was clearly observed. The diphasic spike showed an amplitude increase from 20 sec to about 2 min of anoxia. From this time the amplitude showed a gradual decrease, but part of it was still present after 3 $^1/_2$ min. The negative wave showed a rapid amplitude fall between 20 and 50 sec of anoxia. Then the amplitude remained almost constant (in some experiments it even increased) for about one minute. Then followed a gradual decrease until it usually was abolished after 3—4 min at the usual temperature. The final decrease of the negative wave started simultaneously with the decrease of the diphasic spike from its pre-anoxic value. The restitution of the diphasic spike started almost immediately after re-institution of the oxygen, while the negative wave showed a more sudden amplitude increase after about one minute. In Fig. 7 B and C the records obtained every 20 sec under the anoxia and the restitution period is drawn superimposed. The amplitude decrease of the negative wave was accomplished by the abolition of more and more of the latest part of this wave, *i. e.* the culmination time of this component decreased. On the other hand, following oxygen readministration the culmination time of the negative wave was also now gradually decreased, at first being longer than in any record before or under the anoxia.

Cooling. Fig. 8 illustrates the effect of body cooling upon the CA3 commissural potential. Identical results were obtained by local cooling of the hippocampal cortex by irrigation with paraffin of 8° C. When the hippocampal cortical temperature was lowered, the latency and the duration of all potential components increased. From 32° C to 24° C all parts of the potential were increased and a spike could be elicited on the negative wave (Fig. 8). Below 24° C the potential was depressed. The CA3 commissural potential could be obtained down to cortical temperatures of 16—18° C. The fading of the potential at this temperature seemed to be more related to heart failure than to the conditions of the cortex. This has been inferred from simultaneous electrocardiographic recordings. The CA3 commissural potential was regularly obtained until gross irregularities occurred in heart excitability. At the bottom in Fig. 8 A two superimposed records are shown obtained at the indicated cortical temperatures to illustrate the slower time course of the record obtained at 24° C, the constant amplitude of the negative wave, and the occurrence of spikes on this wave at the lower temperature.

Fig. 8 B shows the effect of anoxia on the CA3 commissural potential at the temperatures indicated. After 8 min of anoxia at 24° C still a great part of the potential remains, while the potential is approximately abolished by a similarly long anoxic period at 35° C (usually the potential was abolished after 4 min). On oxygen readmission the record obtained at 24° C shows a qualitatively good recovery, while the potential at 35° C shows a poorer recovery. Normally 8 min of anoxia at 35° C will prevent the recovery of all potential components except the diphasic spike.

Discussion

Pattern of response. The CA3 commissural potential is clearly different from the CA1 commissural potential. This reflects the main division of the hippocampus in a regio superior, CA1, and a regio inferior, CA2 and CA3, as shown histologically by CAJAL (1911), KOELLIKER (1896) and BLACKSTAD (1956), embryologically by TILNEY (1938), and biochemically by FLEISCHHAUER and HORSTMANN (1957) and COGGESHALL and MACLEAN (1958). The differences between the commissural potential of CA3 and that of CA1 undoubtedly reflects the different pattern of termination as shown by experimental histological technique (BLACKSTAD 1956).

The form of the CA3 commissural potential as observed in the present study is identical with that reported by CRAGG and HAMLYN (1957), who used the same electrode arrangement. It also bears a striking similarity to the potential recorded from the hippocampus by RENSHAW *et al.* (1940) following stimulation of the ipsilateral fimbria, and by GREEN and ADEY (1956) and DUNLOP (1957) in response to stimulation of the dorsal fornix. However, in the three last articles the authors do not state in which field within the hippocampus the recording electrode was placed.

Interpretation of the CA3 commissural potential. The two main parts of the CA3 commissural potential seem to possess fundamentally different properties. The diphasic spike was recorded from the fimbria or near this structure. It had a low absolute refractory period (1.3 msec), and followed repetitive stimulation up to a frequency of 250/sec. Further, it was resistant to local application of nembutal or strychnine sulfate, but slowly depressed by procaine. The diphasic spike was the last component of the CA3 commissural potential to disappear and the first to reappear after periods of cortical unresponsiveness, spontaneous or induced. It could be recorded during total anoxia for at least 10 min, and was also observed when the connection between the fimbria and the CA3 neurons was severed (ANDERSEN 1959). These observations make it probable that the diphasic spike is due to an afferent volley in the commissural fibres of the fimbria.

On arriving to the CA3 region the afferent volley initiates the negative wave. Since the negative wave has the same threshold and absolute refractory period as the diphasic spike it must be due to a process closely related to the activity of the terminal fibres.

The negative wave was recorded with a maximum in the stratum oriens of the CA3 cortical field and showed phase reversal as the electrode was moved through the cell body layer. This suggests that the surface negative wave is associated with the activity of the basal dendrites, or of the cell bodies of CA3 neurons. CRAGG and HAMLYN (1957), using the same electrode arrangement, reported identical distribution of the CA3 potential.

The CA3 negative wave had some properties in common with the negative

wave recorded from the apical dendritic shaft layer of CA1 in response to a commissural volley, and to an excitatory postsynaptic potential (EPSP). These similarities may be summarized as follows: (i) similar form with a steep rising limb and a nearly exponential decay, (ii) the same threshold as for the diphasic fibre spike, (iii) constant duration independent of stimulus strength, (iv) negative polarity recorded extracellularly, (v) ability of summation, (vi) absolute refractory period presumably zero, (vii) moderate susceptibility to urethane, nembutal and anoxia, (viii) high resistance to strychnine, and (ix) apparently causal relation to spike formation. The spike was often only a crest on the summit or on the rising phase of the negative wave, but a well developed spike was recorded when the cortex showed moderately high or high irritability.

The presence of a negative wave was a necessity for the formation of a high amplitude negative wave or a negative spike.

The absolutely refractory period was not longer than that of the diphasic fibre spike. Therefore, it may be suggested that the negative wave itself does not have any absolute refractory period at all, or that it is very short.

The negative wave of the CA3 commissural potential is interpreted as a summated excitatory postsynaptic potential as it has the properties mentioned in common with the EPSP of motoneurons (ECCLES 1957). This suggestion also receives support from anatomical observations. The negative wave was only recorded in the stratum oriens of CA3, the same area in which BLACKSTAD (1956) observed terminal degeneration of interhemispherical fibres. This finding lends support to the interpretation of the negative wave of the CA3 commissural response as an EPSP elicited by the synaptic bombardment of the basal dendrites of the CA3 pyramidal neurons.

Nembutal and urethane, as well as long-lasting tetanic stimulation depressed the late spike and the major part of the negative wave, but a remnant of the negative wave was most often preserved.

The element of the CA3 commissural response which was most susceptible to anoxia was the spike, then followed the negative wave. By plotting the amplitude of this wave against the duration of the anoxia one may divide the negative wave into two functionally different parts (Fig. 7). The major part of this wave disappeared after 50—60 sec af anoxia, while the remaining part showed a fairly constant level to about 3 min, when a decrease of the negative wave simultaneously with the diphasic wave was observed. It is not unlikely that the relatively anoxia-resistant part of the negative wave is identical with the component resistant to nembutal, urethane and fatigue caused by high frequency stimulation. Based upon the observations presented above it is suggested that the negative wave may be divided in two parts. The one which is relatively resistant to nembutal, urethane and anoxia is probably an EPSP, produced in the CA3 basal dendrites, while the other, rising directly from the first, and being more susceptible to the same experimental condi-

tions, represents the asynchronous discharge of the CA3 neurons. This assumption is in agreement with the facilitation of the negative wave of the test response at 8—300 msec and its increase by repetitive stimulation. This facilitation was most evident when the negative wave was of great amplitude (compare Fig. 4 E and F). Increase of the negative wave by repetitive stimulation was also reported by CRAGG and HAMLYN (1957). Similarly, the negative wave produced by stimulation of the dorsal fornix is facilitated by paired stimuli at intervals similar to those effective in commissural stimulation, while the preceding diphasic spike remained unaltered (GREEN and ADEY 1956).

A potential pattern similar to the CA3 commissural potential has been reported from the lateral geniculate body after optic nerve stimulation (BISHOP and MCLEOD 1954). A sharp wave, interpreted as the firing of the geniculate neurons, rises without discontinuity from a negative wave.

The interpretation of the negative wave of the CA3 commissural potential as the synaptic activation of structures lying near the surface is in accordance with similar conclusions arrived at by KAADA (1951, p. 31) studying the primary olfactory area in response to olfactory bulb stimulation and by BUSER (1955, 1956) working with the response of the optic lobe of certain lower vertebrates to optic nerve stimulation.

The small notch in front of the negative wave may be due to the invasion of antidromic impulses into the CA3 cell bodies, or to the activity of fibres with slower conduction velocity than those responsible for the diphasic spike. This assumption is based upon its high resistance to anoxia, nembutal and urethane. At present it is difficult to decide which of the two possibilities is the most probable one. It is expected that the afferent volley contains relatively few antidromic impulses as the threshold of the cell bodies is lower than the threshold of the thin fibres of the fimbria (ANDERSEN 1959).

The interpretation of the negative wave as a prolonged negativity of preterminal fibres seems improbable on account of the discontinuity between the diphasic spike and the negative wave and also because of the total abolishment of the negative wave by anoxia, nembutal, urethane and fatigue.

Regarding the later components of the CA3 commissural potential, the positive wave with the small spikes, these are clearly of postsynaptic and probably of polysynaptic origin. Because of the many histologically possible pathways their further exact interpretation presents great difficulties.

The negative spike recorded from 1.6—2.0 below the surface may be due to the activity either of the granule cells or of other cellular elements in the area dentata or to the activity of deeply situated CA3 neurons. The dense intermingling of various cell types, especially of their dendrites, in this part of CA3 and the dentate area makes the interpretation of responses obtained by a recording electrode from this region difficult. Even after a number of experiments it is still uncertain which of the structures mentioned above are responsible for this spike. Also the relation between this deep negative spike and the

little notch in the start of the surface negative wave is not clear. It is possible that the later part of the surface negative wave of the commissural CA3 potential in part may be due to disynaptic activity (commissural volley — granule cells — mossy fibres — CA3 and CA2 neurons), but the start of the surface negative wave occurs too early (Fig. 1 F and G) to be explained in this way. The smallest time difference between the point of steepest rising of the surface diphasic spike and the start of the deep spike was 0.6 msec. This may probably be taken as the synaptic delay of the synapses in question. The smallest delay between the point of the steepest rising of the diphasic spike and the start of the surface negative wave was 0.8 msec. The short time difference between the deep spike and the surface negative wave when elicited by a common synchronous volley, as low as 0.2 msec, does hardly allow for a conduction along the thin (less than 0.5 μ), unmyelinated mossy fibres for at least 1.5—2.0 mm. It, therefore, seems unlikely that the surface negative wave is the result of firing of the deep spike only, *i. e.* that it should be a purely disynaptic potential. This is also inferred from its short or lacking absolute refractory period. Most probably, both the CA3 surface negative wave and the deep CA3 negative spike are postsynaptic activities directly initiated by an afferent volley through commissural fibres, the later probably by thicker fibres than the former. The highest degree of myelinization of the interhippocampal fibres is found from the base of the fimbria into the hilus of the dentate fascia (Allen 1948, Andersen 1960 b).

The large positive wave recorded deep in CA3 and in CA4 is also difficult to explain. It is recorded too constantly and over too big an area to be interpreted as a sign of the activity of deteriorating neurons. It may be due to the synchronous activity of the CA3 neurons. The prominent amplitude increase by an electrode displacement of only 10 μ suggests an origin from a restricted area.

Effect of anoxia. The depressant effect of anoxia on the commissural responses of both CA1 and CA3 followed a similar time course as the corresponding effect on the focal synaptic potentials elicited in the spinal cord by the monosynaptic stretch reflex (Brooks and Eccles 1947) and the effect on the dorsal spinal cord potential (Gelfan and Tarlov 1955). This observation suggests that the initial parts of both the CA1 and the CA3 commissural potentials are due to monosynaptic activation.

Brooks and Eccles (1947) ascribed the effect of anoxia on the monosynaptic stretch reflex to a depolarization of the presynaptic, terminal fibres and of the postsynaptic soma membrane. However, the presynaptic terminals were blocked before the soma membrane as judged by experiments with antidromic activation of the motoneurons. This finding lends support to the assumption that the small negative crest at the beginning of the negative wave of the CA3 commissural potential is due to the antidromic activation of the pyramidal cells. This crest was more resistant to anoxia than the rest of the negative wave.

The initial stage of increased excitability during the anoxia is most probably related to the anoxic depolarization (LORENTE DE NÓ 1947). Similar increased activity as the result of short-lasting anoxia has been reported for a variety of neuronal processes, *e. g.* spontaneous brain waves, the transmission in central and peripheral autonomic synapses, spinal reflexes and peripheral nerve fibres (for references see BROOKS and ECCLES 1947).

The explanation of the post-anoxic hyperirritability offers greater difficulties. It may be explained as a release phenomenon. Thus, if the hippocampus contains interneurons which are inhibitory in nature, these secondarily activated interneurons, would probably be blocked longer than the directly activated neurons by an anoxic period. This assumption is similar to the explanation of the delayed increase of the monosynaptic reflex responses of the spinal cord exposed to oxygen lack (KIRSTEIN 1950). However, in nerves and in monosynaptic transmission the rule is that a fairly long-lasting depression develops when oxygen is readmitted after a period of anoxia (BROOKS and ECCLES 1947, KIRSTEIN 1950). This is probably related to the post-anoxic hyperpolarization of the nerve cell membrane (LEHMANN 1937, LORENTE DE NÓ 1947).

When the anoxia produced afterdischarges the restitution following oxygen readmission was greatly delayed, indicating that the afterdischarges had produced profound biochemical changes in the neurons.

Effect of cooling. The increase of the surface negative wave of the CA3 commissural response, especially of its duration with increased neuronal discharge, at lowered temperatures is in agreement with the interpretation of this wave as a summated EPSP. Thus, BROOKS, KOIZUMI and MALCOLM (1955) found that cooling of the spinal cord to 25° C increased focal synaptic potentials in amplitude as well as in duration. In cat muscles, blocked by magnesium, the endplate potentials are augmented both in amplitude and duration following lowering of the temperature (BOYD and MARTIN 1956). Further, STOVNER (1957) observed release of magnesium block of the rat diaphragm on decreasing the temperature.

The present experiments give little information regarding the mechanism of the cold hyperirritability. However, as all parts of the CA3 commissural potential were increased to the same degree, including the diphasic fibre spike, it seems that the effect is not only located to a restricted structure, *e. g.* the presynaptic terminals, but also to the afferent myelinated nerve fibres.

Effect of repetitive stimulation. The marked facilitation of the CA3 commissural response which may last for several minutes after tetanic stimulation is probably an example of post-tetanic potentiation (PTP). The degree of the facilitation was proportional to the frequency and the duration of the repetitive stimulation, as is usual in PTP (HUGHES 1958). The facilitation did not affect the diphasic spike, but as the site of action of PTP probably is in the presynaptic terminal fibres only, this is to be expected.

Differing physiological properties of CA3 and CA1 neurons. On commissural stimulation spikes were more seldom observed in CA3 than in CA1. This observation may be correlated with the different pattern of termination of interhippocampal fibres. The direct commissural influence upon the CA3 pyramids is exclusively on their basal dendrites. These are thinner than the apical dendrites of CA1 with which the commissural terminal fibres to that region synapse. However, the amount and concentration of commissural synapses on the two types of dendrites are unknown. This makes an evaluation of the relative effectiveness of the basal and apical dendritic activation impossible. Another explanation is that the CA3 neurons are under the inhibitory influence from some unknown source, perhaps the dentate area.

The marked difference in the strychnine effect on the CA1 and the CA3 commissural responses is of interest. BRADLEY, EASTON and ECCLES (1953) has shown that strychnine inhibits the inhibitory post-synaptic potential of motoneurons. If a similar mechanism is present in the hippocampus the presented findings of strychnine application might be explained by assuming a continuous inhibitory influence on the CA1 neurons, but none or a small inhibition of the CA3 pyramids. This assumption is admittedly purely hypothetical and the problem needs the use of intracellular recording to be solved. Further, more than one mechansim is certainly involved in strychnine facilitation (BREMER 1953, ECCLES 1957).

As mentioned above, the presence of inhibitory interneurons in CA1 only may explain the post-anoxic hyperirritability. This condition was more often observed in CA1 than in CA3. Further, the assumption of more interneurons activated by the commissural volley in CA1 compared with CA3 receives support from the observation that tetanic and post-tetanic potentiation is easier elicited in CA1 than in CA3. These effects affected the late part of the response only, leaving the initial, probably directly activated part more or less unchanged.

No distinct differences in susceptibility to anoxia were noted between the commissural responses obtained from CA3 compared with those from CA1. This suggests that the described lowered resistance to anoxia of the Sommer's sector, approximately corresponding to CA1, is not due to different anoxic resistance of the neurons, but may be sought in peculiarities of the vascular supply. Such evidence has been put forward by SPIELMEYER (1925) and SCHARRER (1940).

Summary

1. The response of the hippocampal field CA3 to a commissural volley has been analysed in cats, rabbits and rats under urethane-chloralose anesthesia. The CA3 commissural response consisted of a diphasic spike followed by a

negative wave, sometimes with one or two superimposed spikes. Following stronger stimulation a late positive wave with small spikes developed.

2. The diphasic spike had the same threshold as the negative wave. The spike had a short absolute refractory period (1.2 msec), was resistant to high frequency stimulation, to strychnine, nembutal and urethane. It resisted anoxia for 10—15 min. The diphasic wave is interpreted as the sign of impulse conduction in commissural fibres in the fimbria approaching and entering the field CA3.

3. The negative wave was recorded only from the layer of the basal dendrites of the CA3 pyramidal cells. It had the same threshold and absolute refractory period as the diphasic spike, the ability of summation, and was markedly influenced by repetitive stimulation. It showed post-tetanic potentiation and sometimes a post-tetanic depression. It was little, if at all affected by strychnine, but it was depressed by urethane and pentobarbital. It resisted anoxia for 4—5 min at a local hippocampal temperature of 35° C. The negative wave consisted of two parts differing in their susceptibility to anoxia. The negative wave is probably partly an excitatory postsynaptic potential produced in the basal dendrites of the CA3 neurons by the commissural impulses, and partly an asynchronous postsynaptic discharge of these cells.

4. The spike riding on the summit of the negative wave had a higher threshold than the negative wave, a longer absolute refractory period, and was more susceptible to urethane, nembutal and anoxia than was the negative wave. This spike is probably due to synchronous CA3 neuron discharge.

5. Depth recording revealed a negative spike of great amplitude near the border between the CA3 and the dentate area. It had the same, or slightly shorter latency than the surface recorded negative wave. It is still uncertain whether it is due to deeply situated CA3 neurons or to dentate granule cells. Both the surface negative wave and the deep negative spike is probably produced by direct commissural activation, the later possibly through the thickest fibres of the commissure.

6. The same commissural volley elicited spikes less effectively in CA3 than in CA1 where apical dendritic activation is the most prominent. The CA3 commissural response was only slightly influenced by strychnine while the late part of the corresponding CA1 potential was markedly augmented. The susceptibility to anoxia of the CA3 and CA1 commissural responses was similar. The alleged increased vulnerability of the Sommer's sector (CA1) to anoxemia may probably be explained by peculiarities of the vascular supply.

7. Anoxia often produced electrical afterdischarges. Under these circumstances the restitution from even short-lasting anoxic periods was markedly increased compared to anoxic periods which did not provoke afterdischarges.

8. Cooling of the cortex produced increase of the amplitude of all parts of the potential between 32 and 24° C. In the same temperature range a negative spike appeared on the negative wave. The duration of all components

and their latencies increased. The anoxic survival time increased with lowered cortical temperatures. By continuous lowering of the body temperature the CA3 commissural potential was recorded till heart failure occurred (at 16—18° C).

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Acta physiol. scand. 1960. 48. 231—237

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The Blood Lactate During Recovery from Sprint Runs

By

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Abstract

Andersen, K. Lange, A. Bolstad and S. Sand. *The blood lactate during recovery from sprint runs.* Acta physiol. scand. 1960. *48.* 231—237. — The time required to restore the normal resting level of blood lactate after runs of distances up to 800 metres was determined. The recovery time increases as the distance becomes longer and up to a limit of about 400 m. Longer runs do not increase the recovery time for blood lactate. Running 400 m is also sufficient to produce the maximum blood lactate, which in this study was found to range from 120 to 150 mg %. Determination of pulmonary ventilation during recovery was undertaken, and it was found that pulmonary ventilation reached stable values of about resting level long before the excess lactic acid was removed from the blood. Measurement of O_2-debt after the runs revealed a larger debt for an athlete than for a non athlete, and evidence was found indicating a greater "alactic" O_2-debt in the athlete than in the non athlete.

It is obviously of great importance to know the time required for the body functions to recover after performance of muscular exercise. Although numerous investigations have been carried out in the laboratories to study this problem, surprisingly little evidence is available showing the recovery time for most of the daily life activities, including the usual types of athletic exercises.

Here will be presented data showing the time required to restore the normal resting level of blood lactate after runs of distances up to 800 m. In addition pulmonary ventilation and oxygen debt have been studied in a few experiments.

Methods and Experimental Procedures

The experiments were performed on an athletic ground late in the autumn. The weather conditions were sometimes unfavorable for obtaining maximal performances; however, the wind velocity exceeded 1.3 m/sec in only one experiment, and the air temperature ranged between 5 and 15° C.

Healthy young men were used as experimental subjects, most of them being students at the college of Physical Education in Oslo. They were all well motivated and experienced for this type of running and did their best, though the runs were arranged separately and without the extra stimulus usually associated with a competition arrangement. The running times recorded were close to their earlier optimal scores.

Capillary blood was taken from the ear lobe at regular intervals after cessation of the run, the subjects being at rest in a semirecumbent position. Blood lactate was determined colorimetrically by using STRÖM's modification of Barker and Summerson's method (1949).

In a few experiments the pulmonary ventilation was measured by having a respiratory valve of the ENGHOFF type (1930) connected to the subject and passing the expiratory gas volume through a calibrated dry gas meter. Thus the expiratory volume was recorded. Though this does not exactly equal the inspiratory volume, the difference is small and for this purpose negligible.

The oxygen debt was determined by collecting the expired gas volume in Douglas bags for a period of 20—30 min after the runs. Small gas samples were taken from the bags and analyzed for O_2 and CO_2-content by using SCHOLANDER's 1/2 ml gas analizer (1947).

Oxygen intake at rest was determined in a similar way in a warm environment with the subjects in the same semirecumbent position. The resting value for oxygen intake was subtracted from the total oxygen intake during the recovery, the difference being the oxygen debt.

Results

Fig. 1 gives the recovery curves for lactic acid and pulmonary ventilation after performance of 100 m sprint run for a well-trained athlete.

The pulmonary ventilation after running 100 m reaches stable values of about resting level in approximately 10 min. At that time the lactic acid is still very high, which shows that the lactic acid as such cannot be the cause of the elevated pulmonary ventilation and confirms observation made by ASMUSSEN and NIELSEN (1946). From then on the pulmonary ventilation drops further and remains on a lower level for about 10—12 min. In this period some of the excess CO_2-output which undoubtedly was blown off immediately after exercise, is retained.

After running 200 m the pulmonary ventilation needs about the same time to reach resting level.

Fig. 2 and 3 give the recovery curves for the blood lactate after performance of 100—200—400 and 800 m runs. The data are plotted on semilogarithmic graph paper. In accordance with several previous reports it was found that the blood lactate increases after exercise, reaching a maximum level 5—8

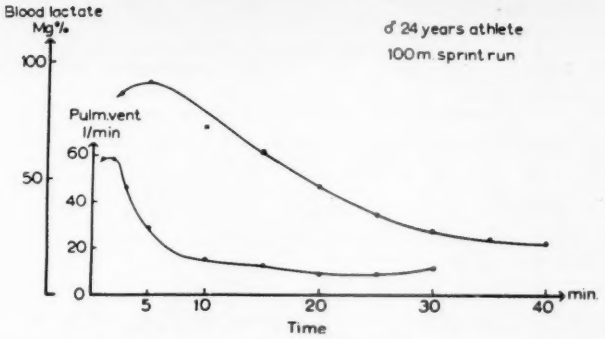

Fig. 1. Blood lactate and pulmonary ventilation (ATPS) during recovery from 100 m sprint run.

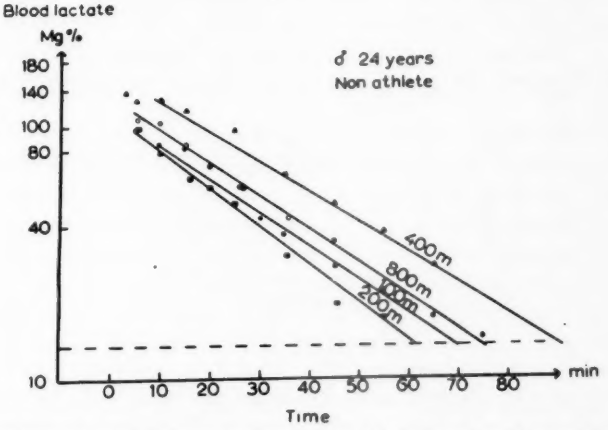

Fig. 2. Blood lactate during recovery from runs up to 800 m. Data plotted on semilogarithmic graph paper.

min after cessation of the exercise. From then on the recovery curve has a downward trend which is exponential against time, and consequently a recovery time can be fixed accurately.

The recovery time increases as the distance becomes longer and up to a limit of about 400 m. Longer runs do not increase the recovery time for blood lactate.

The maximum blood lactate observed for 7 male subjects is shown in Fig. 4. It is apparent that running 400 m is sufficient to produce the maximum blood lactate, which in this study was observed to range from 120 to 150 mg %.

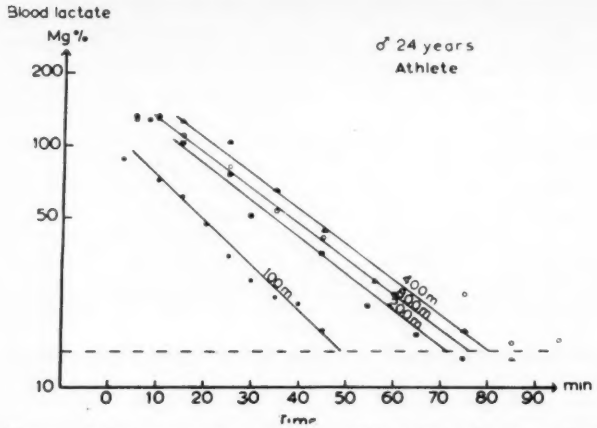

Fig. 3. Blood lactate during recovery from runs up to 800 m. Data plotted on semilogarithmic graph paper.

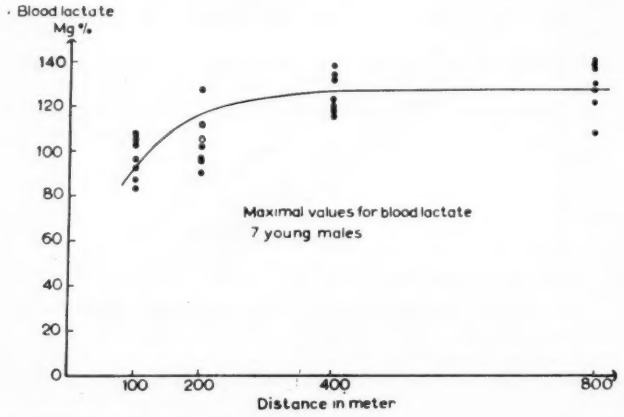

Fig. 4. Maximal values for blood lactate.

In Table I are listed the O_2-debts for the types of exercises studied, which for some of the runs were determined simultaneously with the blood lactate. The O_2-debt is for all distances higher for the athlete than for the non athlete.

Discussion

ERIKSON (1957) investigated the respiratory gas exchange after 70 yards sprint runs and found recovery times of up to 16 min. Oxygen debt was in most of the experiments paid off before the CO_2-output reached the resting

Table I. O_2-debt after running in an athlete and a non athlete. Volumes given in litres at STPD.

Distance in metres	Athlete	Non athlete
100	9.2	4.9
200	9.7	—
400	14.2	7.1
800	13.9	11.6

level, and the pulmonary ventilation followed closely the CO_2-output. Subresting values for CO_2-output were observed during the later recovery period, showing a CO_2-retention.

In this study the pulmonary ventilation after 100 and 200 m runs was recorded. Stable values of about resting level were obtained approximately 20—25 min after the 100 m and 30—35 min after the 200 m run. The removal of lactic acid from the blood was found to be a slower process in accordance with other reports in the literature (BARMAN, CONSOLAZIO and MOREIRA 1942, ANDERSEN 1959).

The oxydation of lactic acid to CO_2 and H_2O and the resynthesis to glycogen are processes which require oxygen. These reactions take place mainly in the liver, the energy production of which would thus be increased as long as the blood lactate is elevated, presuming that the rate of the metabolism of carbohydrate, fat and protein is unchanged. However, this generally accepted assumption may be wrong. If so, the total oxygen intake may be at the normal resting level before the surplus of lactic acid is removed from the body. There is some evidence in the literature for this hypothesis. As previously referred to, BARMAN *et al.* (1942) and ANDERSEN (1959), found that the oxygen intake reached the normal resting level before the blood lactate.

As previously pointed out the recovery time for lactic acid can be determined accurately by plotting the data on semilogarithmic graph paper. Such an accurate determination of the recovery time for respiratory gaseous exchange is difficult to undertake, as the recovery curve for the gas exchange may deviate considerably from the pure logarithmic curve (SIMONSEN 1926, ANDERSEN 1959). This is also apparent from the data presented here.

The oxygen debt which was determined in one athlete and one non athlete, revealed large interindividual differences with lower values for the latter. This shows that the energy output caused by anaerobic processes is greater for the athlete than for the non athlete. Realizing that the amount of lactic acid produced (which can be roughly calculated from the maximal blood lactate and the body weight on the assumption that the lactic acid is equally distributed in the body) is about the same in the athlete and the non athlete,

it appears that the "alactic" oxygen debt is greater in the athlete than the non athlete. However, the experimental evidence here presented is too scarce to permit any safe conclusion, and further studies directed on this topic should be undertaken for verification. It is generally accepted (HILL, LONG and LUPTON 1923—1924, MARGARIA, EDWARDS and DILL 1932, PEARL, CARLSON and SHERWOOD 1956) that the mechanism for the oxygen debt is the oxidation of anaerobic metabolites and the resaturation of muscle myoglobin with oxygen. However, as far as seen from the available literature, very little is known about the muscle tonus after exercise. This may well be increased for a considerable period of time following completion of exercise, and this mechanism will also most likely result in elevated oxygen intake, and thus be a part of the oxygen debt.

A 400 m sprint is apparently sufficient to draw upon the total anaerobic energy producing reserves in the muscles engaged in running, and the experimental results bring together evidence about the anaerobic working capacity, which is greater for the athlete than for the non athlete.

It was surprising to observe how long it took before the lactic acid was removed from the circulating blood. This knowledge implies practical consequences for the performance of athletics with regard to the time intervals between the starts. It seems reasonable to recommend these intervals to be at least the length of time necessary to recover from the first run. This means 40 min pause between two 100 m starts, which should be increased to at least 75 min for the 400 and 800 m runs.

Gratitude is extended to Statens Ungdoms- og Idrettskontor who supported this study.

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Thalamic Neurones Responding to Tactile Stimulation of the Cat's Tongue

By

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Abstract

LANDGREN, S. *Thalamic neurones responding to tactile stimulation of the cat's tongue.* Acta physiol. scand. 1960. *48.* 238—254. — Encéphale isolé preparations were used. Extracellular spike potentials were recorded from single neurones in Nucleus ventralis posteromedialis of the thalamus. — The response of 40 thalamic neurones to tactile, thermal and gustatory stimulation of the tongue and to electrical stimulation of the tongue and the ipsilateral dorsal ascending trigeminal tract was investigated. Out of these neurones 32 responded only to touch, 4 only to pressure, and 4 to both touch and cooling of the tongue. One third of the neurones showed tactile receptive fields about 2 mm in diameter. Two thirds had larger ipsilateral or contralateral receptive fields. No large bilateral fields of the type shown by cortical "touch cells" were observed. — The typical response evoked in thalamic neurones by touch or electrical stimulation of the tongue was a short burst of spikes. Generally no after-discharge was seen, but repetitive tactile stimulation sometimes caused an increase in the spontaneous discharge. The mean latency of the first propagated spike in the response of a "touch cell" to electrical stimulation of the tongue was generally 3—6 msec (shortest latency 2.5 msec). Most mean latencies of response to tract stimulation were 1—2 msec (shortest latency 0.9 msec).

The activity of single cells in the cortical tongue projection area of the cat was investigated by COHEN *et al.* (1957), and by LANDGREN (1957 a and b). Specific cortical neurones were found, which responded only to touch on the tongue. Other neurones, however, were discharged both by mechanical and by thermal or gustatory stimuli. It was concluded that this was due to convergence within the central nervous system of fibres subserving the different

modalities but the level at which the convergence occurred was unknown. A typical pattern of response, consisting of an initial burst of action potentials, a pause in the activity, and a delayed after-discharge was commonly observed in cortical neurones discharged by touch of the tongue.

It was considered that it would be of interest to study neurones within the thalamic relay nucleus responding to tactile stimulation of the tongue in order to find whether their behaviour differed from that of cortical "touch cells". The results of such an investigation are presented in the following report.

Methods

Twenty-two cats were used for the experiments. The animals were anaesthetized with thiopentone sodium (May and Baker), ether or trilene (ICI) during the operation. Local anaesthesia was applied to incisions and pressure points before mounting the animal in the Horsley-Clarke apparatus. The spinal cord was blocked at the level of C 2 by local injection of Xylocaine (Astra), and the experiments carried out in an encéphale isolé preparation. In a few cases thiopentone sodium anaesthesia was used throughout the experiment, and the spinal cord was left intact.

Glass micropipettes filled with 3 M KCl or 4.5 M NaCl were stereotactically guided to the nucleus ventralis posteromedialis (VPM) of the thalamus, where spike potentials of single neurones responding to stimulation of the tongue were recorded.

Physiological stimuli (touch, pressure, cooling, warming, 0.5 M NaCl, 0.02 M Quinine HCl, 0.1 M acetic acid, distilled water) were applied to the tongue. Electrical stimulation was applied to the surface of the tongue, to the chorda tympani or the trigeminal component of the lingual nerve, or to the ipsilateral dorsal ascending trigeminal tract at the mesencephalic level.

The position of recording and stimulating electrodes was confirmed in histological serial sections.

All records show positive deflections upwards. Further details concerning the recording and stimulating technique are given by COHEN *et al.* (1957), APPELBERG and LANDGREN (1958) and by APPELBERG, KITCHELL and LANDGREN (1959).

Results

As the error of the stereotaxic method of localization was large compared to the volume of the thalamic relay nucleus for the specific sensory path from the tongue, an additional guide was used in placing the tip of the microelectrode within the nucleus. This guide was the negative component of the thalamic focal potential evoked by electrical stimulation of the ipsilateral tip of the tongue or the ipsilateral lingual nerve (cf. APPELBERG and LANDGREN 1958). The observations of unitary activity to be presented in this report were therefore sampled from the population of thalamic neurones situated within the region of the focal potential or in its immediate surroundings. The choice of the area to be investigated rests on the interpretation of the negative evoked potential as a sign of post-synaptic activity within the thalamic relay nucleus. It is, however, possible or even likely that this potential is due to activity in the relay cells of the tactile path only and does not tell us anything about the localization of the relays in the thermal or gustatory paths. It was determined histologically that the region investigated was the *nucleus ventralis posteromedialis* (VPM) of the thalamus (cf. APPELBERG and LANDGREN 1958).

Table I

Type of stimulus	Receptive field	Number of cells with:		
		Ipsilateral receptive field	Contralateral receptive field	Bilateral receptive field
Touch	Tongue	31	8	1
Pressure	Tongue	3	0	1
Touch	Mouth cavity excluding tongue	6	1	1
Touch	Face (lips, nose, whiskers and chin	2	12	3
Touch	Forepaw	0	6	0

The material consists of records and observations obtained from 94 thalamic neurones responding to tactile stimulation, pressure, or stretch, applied to the tongue, the mouth and neighbouring areas of the face, and in a few cases to the contralateral forepaw. Most of the units investigated (viz. 63) had their receptive fields on the tongue.

A. The tactile receptive field of the thalamic neurones

The receptive fields of the thalamic neurones responding to tactile stimulation of the tongue were all found within the anterior two thirds of the tongue. They were generally easy to define and localized either to the ipsilateral or contralateral side. Receptive fields extending across the midline were very rare. In fact only one "tongue cell" out of 40 showed this feature. It responded to touch to the contralateral tip of the tongue but also to a small area of the ipsilateral tip close to the midline. Two out of 17 "face cells" had a bilateral receptive field. Most of the investigated neurones, viz. 70 %, had their receptive fields on the tip of the tongue. These fields did not extend beyond the anterior third of the tongue. About 20 % of the neurones responded to touch of the lateral edge within the middle third of the tongue, and about 10 % showed receptive fields within the area covered by the dorsal papillae. One third of the observed cells showed small well-defined receptive fields with a diameter of about 2 mm. These observations are comparable with those of COHEN *et al.* (1957) on the receptive fields of tactile cells within the cortical tongue projection area. In the present material, however, one type of receptive field seems to be missing i. e. that of the cortical cells responding to touch to both sides of the tongue or to the tongue and the nose. Thalamic neurones never showed such large receptive fields.

The observations concerning the ipsi- or contralateral connections of the thalamic neurones are summarized in Table I.

It is well-known that the mouth is bilaterally represented at the thalamic level (cf. MOUNTCASTLE and HENNEMAN 1949). The proportions of ipsilateral and contralateral observations are, however, interesting. Table I shows that

the majority of responses from the tongue and mouth cavity were ipsilateral, whereas those from the perioral regions were mainly contralateral.

The observations made on thalamic neurones responding to tactile stimulation outside the tongue were included in this material in order to define the borders of the tongue relay nucleus. These cells were found in tracks penetrating just outside the region of the evoked tongue potential. They sometimes occurred along the same track as a number of tongue neurones. As the microelectrode was advanced in steps of only a few microns, a considerable degree of accuracy may be attributed to observations on relative localization of cells found along the same track. It is therefore of interest to note that tongue neurones were always grouped together along the same track and were never mixed with cells responding to touch of the oral mucosa or perioral region. It was also consistently observed that neurones with receptive fields within the maxillary region were situated dorsally to the tongue neurones in the same track. Similarly responses from the mandibular region were always obtained ventrally to those from the tongue. Tracks along which face and tongue neurones were found lay laterally to those showing tongue responses only. The small number of cells responding to stimulation of the contralateral forepaw were found ventral to face or tongue responses. Neurones in tracks penetrating just medially to the region of the evoked tongue potential did not respond to any of the applied stimuli. These observations indicate a very precise topographical localization of the tactile tongue relay nucleus within VPM. However, thalamic neurones with ipsilateral and those with contralateral receptive fields do not seem to be separately grouped within the tongue relay nucleus, as responses from such neurones were recorded only a few microns apart along the same microelectrode track.

B. The tactile specificity of the thalamic neurones

In the present material 40 thalamic neurones were fully tested with tactile stimulation, cooling, warming and flavoured solutions (salt, bitter, acid and water) applied to the tongue. Out of these neurones 32 responded to light touch and 4 to pressure on the tongue but not to any thermal or gustatory stimuli. Four cells responded to touch and cooling but not to warming or to flavoured solutions. It was difficult to decide whether a tactile response and a response to cooling were obtained from one and the same cell. Fairly often the activity of two or more neurones was recorded from the same microelectrode site. Seven observations of combined touch and cold responses were discarded as the records showed action potentials of more than one type. In the above mentioned four cases responding to touch and cooling the high signal-to-noise ratio permitted a careful comparison between the spikes of the tactile and the thermal response. They showed a good agreement in shape, amplitudes and spontaneous frequency and it was therefore concluded that they originated from the same neurone. A combined response to touch and salt solutions was

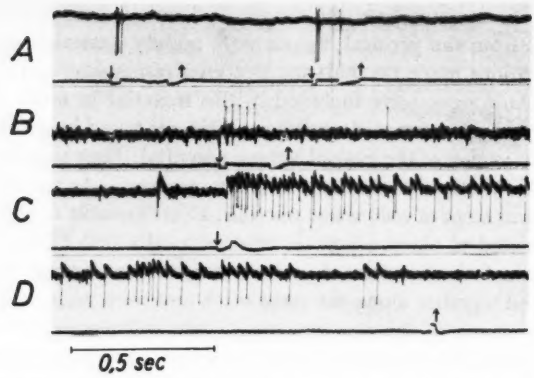

Fig. 1. Response pattern of thalamic neurones discharged by light touch (A and B) and by pressure (C and D) on the tongue of the cat. D is continuation of C. Arrows mark beginning and end of stimulus. Positive deflections are recorded upwards in this and the subsequent figures.

recorded in 3 cases, but the records were discarded as they showed more than one active unit. The touch and the taste responses may therefore have originated in two different cells.

Some of the neurones responding to touch of the lips or nose were also tested on their reaction to cooling and to warming of the receptive field. The number of cells thus tested were 9, and none of them responded to thermal stimuli. In one case responses to touch and cooling of the nose were recorded, but these observations were discarded because the records showed more than one spike amplitude and it was impossible to prove whether the responses originated from the same cell.

Summing up the results presented above one can thus say that 90 per cent of the material consists of cells accessible to tactile stimuli only. However, thalamic cells responding to more than one type of stimulus do exist.

C. The pattern of activity in response to physiological stimulation

All the thalamic neurones investigated were spontaneously active. The frequency of this activity was generally low and irregular and it was therefore easy to recognize responses to tactile stimulation.

The response of the thalamic neurones to touch was of short latency and consisted of a short burst of spikes. This type of rapidly adapting discharge was typical for the thalamic cells observed which responded only to light touch of the tongue. It is shown in Fig. 1 A and B. The latency was generally 8 msec (range of observations 4—20 msec). Each burst of response consisted of 1 to 20 spikes discharged during less than 100 msec. The highest observed frequency averaged over the length of the burst was 625 impulses per sec. The duration of the discharge was generally shorter than the period of stimulation,

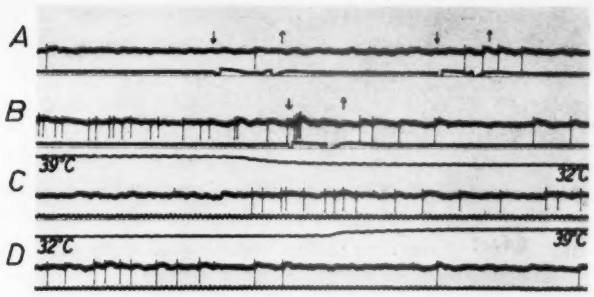

Fig. 2. Records from a thalamic neurone responding to touch (A and B) and to cooling (C and D) of the tongue. A and B show beginning and end of a period of repetitive tactile stimulation of the tongue (1 per sec). The interval between records A and B was 22 sec. Tactile stimulation signalled by deflections on lower beam and indicated by arrows. D is a continuation of C. Thermode temperature signalled on top beam. Time: 50 cps.

the beginning and end of which is marked by the arrows on the records of Fig. 1. The cells were often discharged both by the onset and the release of the touch thus showing a kind of "on-off response" as seen in Fig. 1 A (the second of the tests). However, the "off response" was irregular, owing probably to the uncontrolled type of stimulus used. As a rule no after-discharge was observed in these neurones but repeated tactile stimulation could induce an increase of their irregular spontaneous activity.

A few thalamic cells showed a slowly adapting response as seen in Fig. 1 C and D. This record was obtained from a neurone activated by pressure within a small well-localized area of the ipsilateral tip of the tongue. The cell was also discharged by transverse stretch of the tongue but not by longitudinal stretch, light touch, cooling, warming or taste. It responded to continuous pressure with a slowly adapting series of spikes lasting for as long as the stimulus was applied. Similar tonic responses were recorded from a few other thalamic cells activated by movement of the mandible or flexion of the toes on the forepaw. The receptors were probably stretch-receptors in muscles, tendons or joints.

A third type of unitary discharge evoked by tactile stimulation of the tongue is shown in Fig. 2 A and B. Neurones of this type responded to a single stimulus with one or a few spikes. The latency was long and irregular and the frequency of the discharge was low (Fig. 2 A). If the receptive field was repeatedly touched the spontaneous activity of the thalamic cell was gradually increased. The latency of the response to each touch was shortened and the impulse frequency of the discharge increased. The effect is shown in Fig. 2 B, which was recorded 22 seconds after A during a series of repeated touch stimuli to the tongue at a frequency of about 1 per sec. The small number of cells discharged both by touch and by cooling of the tongue showed a tactile response of this type. The thalamic neurone seen in Fig. 2 responded promptly to cooling.

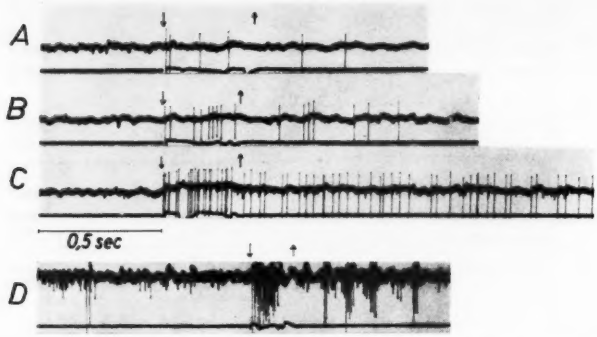

Fig. 3. Records from thalamic neurones responding to pressure on the chin (A—C) and to touch of the tongue (D). A—C are consecutive parts of the same record. Beginning and end of tactile stimulation marked by arrows. Note the slowly adapting response to a brief stimulus and the increase in discharge with repeated stimulation. Record D shows rhythmic after-discharge.

Its reaction to a change of the tongue's temperature from 39 to 32 and back to 39° C is illustrated in Fig. 2 C and D.

After-discharge — i. e. a series of late discharge lasting considerably longer than the phasic tactile stimulus — was only seldom recorded. Such a pattern was, however, seen in 5 cells. It may be described as a fourth type of thalamic response to mechanical stimulation. Fig. 3 A—C shows this pattern of discharge. The neurone responded with a series of action potentials to pressure below the chin. The length of the series and the frequency of the discharge was greatly increased by repeated touch (see Fig. 3 B and C) and lasted considerably longer than the short touch stimulation indicated by the arrows and by the deflections of the lower beam. This neurone showed a spontaneous activity consisting of groups of spikes similar to those triggered off by the touch.

Similar responses were recorded from three cells with their receptive fields among the contralateral whiskers. A brief touch of the whisker area resulted in long-lasting series of discharge appearing with the comparatively long latency of 200 to 700 msec. These three cells were found along the same track. Injection of d-tubocurarine did not change their response.

The after-discharge described above adapted slowly but showed no regular grouping of the spikes in rhythmically appearing bursts. A rhythmic after-discharge was seen only once. It was found in a record with several cells, which usually responded with a short burst of spikes to touch of the contralateral tip of the tongue. Some of the tests, however, elicited a rhythmic series of bursts appearing with a frequency of 10 per sec. The response is shown in Fig. 3 D. The cells were spontaneously active at a rather high frequency and spontaneous bursts of discharge were seen.

D. The response to electrical stimulation

1. The action potential of the thalamic neurone

Recording from cortical cells responding to touch of the cat's tongue COHEN *et al.* (1957) observed that the extracellularly recorded spike changed from a negative to a diphasic type when the cell was approached with the microelectrode. These changes were carefully analyzed by PHILLIPS (1959) in experiments on antidromically activated Betz cells. The change from a negative to a diphasic extracellularly recorded spike and finally to an intracellularly recorded positive action potential was clearly demonstrated when the microelectrode approached the Betz cell. In the present series of experiments all these types of cellular potentials were met with. Only the extracellularly recorded spikes were, however, stable enough to be analyzed.

ROSE and MOUNTCASTLE (1954) observed that the diphasic spikes of the thalamic cells were generated on top of prepotentials. The prepotential was further studied by FREYGANG (1958) and by BISHOP, BURKE and DAVIS (1958) and it was shown to rise in two steps. The spikes of the thalamic neurones recorded in the present series of experiments were similar to those described by the authors mentioned above, and only some details will be described and discussed here.

In Fig. 4 A and B negative spikes are shown rising from negative prepotentials. Diphasic spikes, however, showed positive prepotentials as seen in Fig. 4 C—E. A prepotential rising in two steps was observed in some of the cells (Fig. 4 F—H). In many cases, however, only one step was found. The initial stage of the prepotential was probably too small to appear above the noise level. The record F of Fig. 4 was obtained from a thalamic cell discharged by electrical stimulation of the dorsal ascending trigeminal tract and the shock artefact causes a considerable distortion of the record. The action potential thus appears on the declining phase of the artefact. Nevertheless the two steps (marked by arrows) may be seen on the rising phase. In records G and H prepotentials of two different amplitudes appear. The smallest one is marked by an arrow, and it is seen preceding the larger prepotential, which in turn occasionally precedes a diphasic spike. The small prepotential also appears in isolation.

In records B, G and H of Fig. 4 a repetitive train of spikes was generated by a single shock delivered to the tract (B) or to the tongue (G and H). The successive prepotentials obviously sum thereby reaching the critical level where a propagated spike is discharged. The first spike often appeared on the second prepotential in the train (B, D and G).

Extracellular and intracellular records obtained from the same neurone were studied by SVAETICHIN (1958) in spinal ganglion cells of the frog and by PHILLIPS (1959) in Betz cells in the cat's cerebral cortex. The extracellular and the intracellular records were compared, and different interpretations of

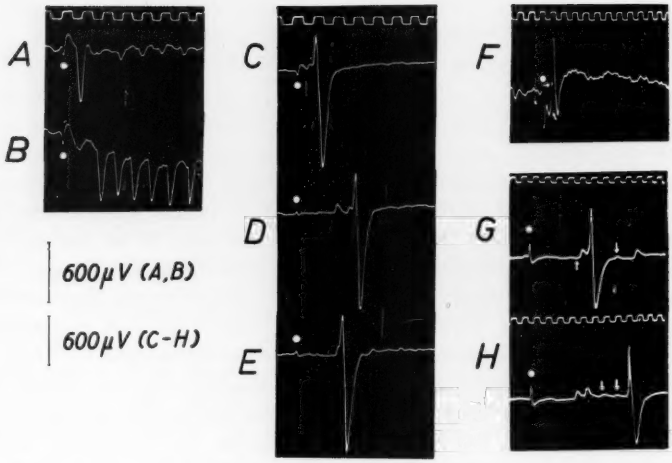

Fig. 4. Extracellularly recorded action potentials from thalamic neurones responding to electrical stimulation of the ipsilateral dorsal ascending trigeminal tract (A, B, C and F) and of the ipsilateral tip of the tongue (D, E) respectively ipsilateral lower lip (G, H). Records A—E obtained while approaching the same cell. Time: 2 msec in all records. A and B are DC records. Amplifier time constant (TC) for records C—F: 50 msec, and for G—H: 500 msec. Positive deflections are recorded upwards. Spikes retouched. Shock artefacts indicated by dots. Arrows indicate the two stages of the prepotential.

the recorded potentials were discussed. The present observations do not contribute anything further to the discussion concerning the origin of the prepotentials and the diphasic spikes, but the observation of prepotentials and of inflexions on the rising phase of the spikes suggest, that these potentials were recorded from the soma-dendritic region of the neurone and not from axones. This is in agreement with the fact, that unitary responses to stimulation of the tongue were always grouped together and never occurred outside the region of the thalamic focal potential evoked by electrical stimulation of the tongue.

Rose and Mountcastle (1954) described a presynaptic potential, which preceded the postsynaptic potentials of thalamic cells. In the present series no certain sign of presynaptic activity was observed in response to electrical stimulation of the tongue. Records of the type shown in Fig. 4 D are similar to those shown by Rose and Mountcastle and it may be tempting to interpret the small initial potential as a presynaptic spike. The following sweep (E), however, suggested that it is more likely to be a prepotential not capable of generating a propagated spike in sweep D (cf. also Fig. 4 G and H). The afferent volley from the tongue arriving to the thalamus via the relays of the medulla is probably too asynchronous to be recorded as a definite presynaptic spike.

It was expected that a presynaptic potential would be found in records from

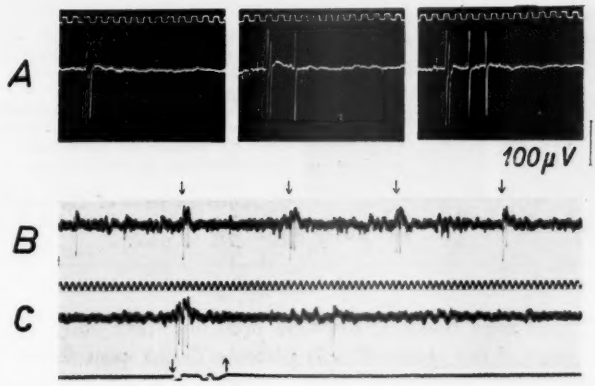

Fig. 5. Response pattern of thalamic "touch cells" discharged by electrical stimulation of the tongue. A. Records showing the variation in number of spikes discharged by a single shock. Time: 5 msec TC 500 msec. Spikes retouched. B. Electrical stimulation of the tongue at 3 per sec. Shocks indicated by arrows. Note no after-discharge. Time: 50 cps., TC 5 msec. C. The cell of record B responding to touch of the ipsilateral tip of the tongue. Touch indicated by arrows.

neurones discharged by electrical stimulation of the dorsal ascending trigeminal tract. Such a potential was demonstrated by BISHOP and MCLEOD (1954) when recording the response to optic tract stimulation in the lateral geniculate body. In Fig. 4 F a small positive deflection, preceding the first step marked with arrow, seems to be superimposed on the approximately exponential stimulus artefact. This deflection could possibly be interpreted as a presynaptic spike, although the artefact makes it difficult to judge.

2. *Pattern of activity*

A single shock electrical stimulation of the tongue surface or the tongue nerves induced a short burst of 1 to 7 spikes in thalamic "touch cells" (Fig. 5 A and B). In 2/3 of the observed cells the burst generally consisted of 2 to 3 spikes but in 1/3 the most regular response was one spike only. Although relatively constant the number of spikes in each burst was not a rigid feature of the neurone's activity. Cells which generally discharged only one spike occasionally responded with a burst of activity, and those usually showing 2 to 3 sometimes discharged up to 7 spikes. It was observed that the number of spikes in the response increased with increasing strength of the stimulus. The duration of a burst was generally about 10 msec but durations from 4 to 20 msec were observed. The impulse frequency within the bursts was high. Values up to 500 impulses per second were noted. These findings agree with those previously reported by ROSE and MOUNTCASTLE (1954).

When discharged by electrical stimulation of the ipsilateral dorsal ascending

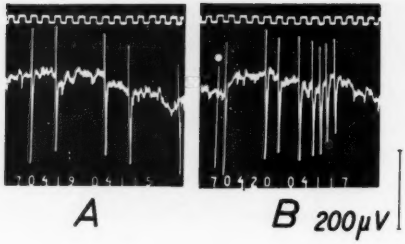

Fig. 6. Records from a thalamic neurone which responded to touch of the ipsilateral palate. A. Spontaneous activity. B. Response to electrical stimulation of the palate. Note the initial spike and the delayed series of discharge. Shock artefact indicated by a dot. Time: 10 msec. TC 50 msec.

trigeminal tract most thalamic neurones fired one spike only. This was true for 75 per cent of the observed cells whereas 25 per cent showed one-spike responses or bursts consisting of 2 to 5 spikes.

A particular point was made of investigating late activity in the neurones. The pattern of discharge was followed in the loud speaker and recorded on a slow sweep (120 msec) or on moving film with a stimulus frequency of 1—3 per sec. An after-discharge was observed in only one cell out of 23. This cell was spontaneously active at a high frequency. It responded to touch of the ipsilateral palate behind the incisors. When the receptive field was stimulated electrically an initial discharge followed by a late group of spikes was recorded. The average length of the pause between the initial and late groups was 28 msec. This pattern of activity may be seen in Fig. 6 B.

3. *Latency of response to electrical stimulation*

The latency of the response of thalamic "touch cells" to electrical stimulation of the afferent path was measured from the stimulus escape to the beginning of the prepotential, and to the point of inflexion which indicated the onset of the steeply rising mono- or diphasic spike potential. The latter component of the action potential was interpreted as a sign of propagated postsynaptic activity. The difference between the two measurements forms part of the synaptic delay. Unfortunately the true synaptic delay could not be measured as no clear-cut presynaptic spike was recorded. The mean latencies of four selected "touch cells" showing well-defined prepotentials are given in Table II. The interval from the beginning of the prepotential to the discharge of the propagated spike varied between 0.4 and 0.9 msec. Similar variations were found whether the neurone was discharged by stimulation of the receptive field or of the dorsal ascending trigeminal tract.

In order to study variations in latency within the available population of thalamic "touch cells" the latency of the first propagated spike potential in the response was measured in 40 thalamic neurones. This latency showed a certain random variation when measured in a series of consecutive records from one and the same neurone. Mean values were therefore computed. The extent of

Table II

Site of stimulation	Latency in msec of		Difference
	prepotential	spike potential	
Ipsi. dors. asc. trig. tract	1.0	1.5	0.5
Ipsi. dors. asc. trig. tract	0.8	1.2	0.4
Ipsi. dors. asc. trig. tract	0.8	1.7	0.9
Receptive field	5.3	5.8	0.5
Receptive field	2.6	3.4	0.8

the variation in latency observed in the individual neurones may be gathered from the following values obtained from two representative thalamic "touch cells".

Responding to electrical stimulation of the tongue the cell of Fig. 5 B showed a mean latency of 4.9 msec (S. D. = 0.98, S. E. = 0.17). The mean latency was computed from a series of measurements made on 35 sweeps. The range of the latencies was 3.8 to 7.3 msec. A series of measurements from another "touch cell" responding to electrical stimulation of the ipsilateral dorsal ascending trigeminal tract showed a mean latency of 1.7 msec (S. D. = 0.17, S. E. = 0.04, n = 18, range 1.5—2.0).

The distribution of the mean latencies of all neurones in this material is illustrated by the histograms of Fig. 7. The histograms A and B of Fig. 7 show mean latencies measured for thalamic "touch cells" with either ipsilateral (A) or contralateral (B) receptive fields. The main group of observations made on ipsilateral neurones is formed by latencies between 3 and 6 msec. The distribution curve tails off to the right showing a small number of rather long latencies.

The histogram showing the latencies of the contralateral neurones comprises a rather small number of observations. The bimodal distribution suggested by the histogram may nevertheless be worth some comment. Records of thalamic focal potentials evoked from the ipsilateral and from the contralateral half of the tongue often, but not always, showed a difference in latency, the contralateral response being the later. Further evidence indicating a longer latency of thalamic cells responding to contralateral stimulation was obtained from experiments with ipsi- and contralateral lingual nerves prepared for electrical stimulation. On the basis of these observations histogram B was expected to show a peak to the right of that seen in A. Such a distribution is also indicated by the column representing latencies between 8 and 9 msec. Most of the contralateral cells, however, responded with short latencies comparable with the main group of ipsilateral observations. The findings therefore suggest that the neurones with contralateral receptive fields may be divided into two

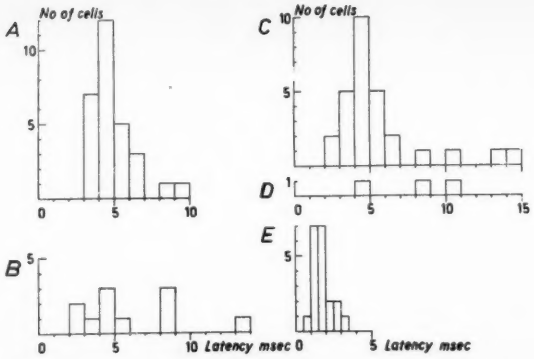

Fig. 7. Histograms showing distribution of mean latencies among certain groups of thalamic "touch cells". All latencies were measured to the onset of the first propagated spike discharged by electrical stimulation of the receptive field (A—D) or of the ipsilateral dorsal ascending trigeminal tract (E). A. Neurones with ipsilateral receptive field. B. Neurones with contralateral receptive field. C. Neurones responding to touch only. D. Neurones responding to touch and cooling. E. Neurones responding to stimulation of the tract.

groups with different lengths of afferent path. This idea is supported by the observation that three of the contralateral neurones with short latency were fired by electrical stimulation of the ipsilateral dorsal ascending trigeminal tract whereas none of those with long latency responded to tract stimulation. Two of the three cells also discharged by the tract had tactile receptive fields extending over the midline to the ipsilateral side. It is therefore probable that cells with bilateral afferent paths contribute to the group of contralateral neurones responding with short latency to electrical stimulation of the tongue.

The distribution of the mean latencies of neurones responding only to touch of the tongue was compared with that of the small number of cells which responded both to touch and to cooling of the tongue. The histogram C of Fig. 7 shows the distribution of the first mentioned group. The other group is represented by the histogram D drawn just below. One cell with short and two with long latencies formed the small group of neurones responding to more than one type of stimulus. The latency of the fourth cell belonging to the group was not recorded. Obviously the number of observations is too small to allow any conclusion.

The histogram E of Fig. 7 shows the distribution of the mean latencies of all observed cells responding to electrical stimulation of the ipsilateral dorsal ascending trigeminal tract. Most latencies were found between one and two msec. A comparison was also made between the distributions of latencies obtained from cells with ipsilateral and with contralateral receptive areas. No significant difference was observed. The same conclusion resulted from comparison between latencies of cells responding to touch only and those of

Table III. Shortest observed latency of response to electrical stimulation

Site of stimulation	Shortest latency in msec	Effective physiological stimuli
Ipsilat. tongue	2.5	Touch of tongue
Contralat. tongue	2.5	Touch of tongue
Contralat. whiskers	2.3	Touch of whiskers
Ipsi. dors. asc. trig. tract	0.9	Touch and cooling of tongue

cells activated both by touch and by cooling of the receptive field. Thus the above-mentioned cell groups could not be differentiated on basis of latency of discharge.

It was further asked whether neurones with short latency to tract stimulation also showed short latency when responding to stimulation of the tongue. This was not the case nor did neurones with long tract latencies constantly show long latency in response to tongue stimulation.

The range of variation of the mean latencies shown in Fig. 7 seems rather large for a homogeneous population of thalamic "touch cells". In particular, the responses to electrical stimulation of the dorsal ascending trigeminal tract were expected to show uniform latencies, as the stimulus was applied directly to the afferent inflow of the thalamic relay nucleus. Although the majority of the observations were found between 1 and 2 msec, as many as 25 per cent of the mean latencies were longer (2—3 msec). A possible explanation of the long latencies could be that the first propagated spike was not fired on the first prepotential but appeared consistently on some of the later prepotentials in the train. However, the possibility also exists that some of the long latencies were recorded from secondary neurones with an additional thalamic synapse in the path.

From certain points of view the shortest observed latency may be of greater interest than the mean latencies given above.

These figures are therefore given in Table III.

An increase in strength of stimulus shortened the latency of the thalamic response. This was also observed in neurones discharged by electrical stimulation of the dorsal ascending trigeminal tract.

Discussion

It was concluded from the present results that the thalamic relays in the tactile path from the tongue are grouped together within VPM and that they were not interspersed in the neighbouring facial relay nucleus. This conclusion seemingly disagrees with the observation of GAZE and GORDON (1954), who found an extensive overlap between the regions of the ventrobasal nuclear complex within which unitary responses to tactile stimulation of the face, the

fore limb, and the hind limb were recorded. The present investigation was, however, mainly concerned with the tongue relay nucleus, and the lateral extension of the face relay nucleus was not investigated. It seems possible that records from axones outside this nucleus were responsible for the overlap observed by GAZE and GORDON.

It may be of interest to compare the responses to tactile stimulation recorded from different levels of the afferent path. The impulse discharge in single "touch fibres" from the cat's tongue was studied by ZOTTERMAN (1936). MARUHASHI, MIZUGUCHI and TASAKI (1952) investigated "touch fibres" from the skin of the cat and also made some observations on "touch fibres" in the lingual nerve. The latter did not differ from those of the cutaneous nerves. It was found that light touch of the tongue elicited a short, rapidly adapting series of large spikes. Pressure fibres responding with a tonic discharge were also observed. Although interesting exceptions were found, it may be stated that the majority of the thalamic "touch cells" responded to touch and pressure in a way similar to that seen in the peripheral nerve fibres. This statement does, of course, not allow the conclusion that the only task of the thalamic neurones is to forward an unchanged signal from the periphery to the cortex. It is, for instance, known that the activity of these neurones may be influenced by the mesencephalic reticular formation (LANDGREN 1959).

As judged from the time relations of their responses, the thalamic "touch cells" described here and the cortical "touch cells" studied by COHEN *et al.* (1957) and by LANDGREN (1957 b) are consecutive neurones in the fastest path from the tongue. The response of the cortical neurones to touch of the tongue did, however, differ considerably from that shown by the preceding thalamic neurones in the path. The cortical response consists of an initial group of spikes discharged with short latency and followed by a period of inhibition lasting about 80 msec. The pause in the discharge during the period of inhibition is followed by a long lasting series of after-discharges. This after-discharge was probably not evoked via the fastest path from the tongue to the cortex, as the phasic discharge of the thalamic "touch cells" was finished long before the beginning of the after-discharge of the cortical neurones. It may, however, be due to activity in polysynaptic side-chains branching off from the fastest path.

The rare occurrence of rhythmical after-discharge in thalamic "touch cells" responding to a brief touch of the receptive field or to a single electrical shock applied to the afferent nerve fibres may be surprising, as such a discharge was observed by ADRIAN (1941) in experiments on anaesthetized animals. It seems possible that the difference is due to the different methods of anaesthesia used in the present experiments. This assumption is in agreement with the experience of GAZE and GORDON (1954), who found that rhythmical after-discharge was rare in thalamic neurones, but also observed, that such a discharge occurred less rarely in Dial anaesthetized preparations.

It is interesting to compare the size of the receptive fields of the peripheral "touch fibres" with those of the thalamic and cortical "touch cells". MARUHASHI *et al.* (1952) thus describe the receptive field of the "touch fibres" from the skin as spotlike and generally consisting of one, sometimes two, touch spots. The pressure fibres studied by these authors also had very small receptive fields (1—2 mm^2). A considerable number of thalamic "touch cells" were discharged from well-defined areas on the tongue surface about 2 mm in diameter. Each of these cells had only one small receptive area. Cortical "touch cells" with similar receptive fields were described by COHEN *et al.* (1957). The spatial discrimination in such a system of neurones depends on the size of the area from which the peripheral neurone can be affected, and nothing is lost in precision during the transmission up to the cortical level. However, the majority of the thalamic "touch cells" in the present material had receptive fields considerably larger than those of the peripheral neurones and still wider tongue areas affected some of the cortical "touch cells". The tactile path from the tongue to the cortex therefore seems to possess two different systems of neurones: one with point to point connections and a minimum of convergence and another system with an increasing degree of convergence at the consecutive levels of the path.

Neurones responding to more than one type of stimulus have been observed at all levels of the fastest path from the tongue to the cortex. Peripheral "touch fibres" also responding to cooling of the cat's tongue were thus described by HENSEL and ZOTTERMAN (1951) and by DODT (1953), and cortical neurones discharged both by tactile and also by thermal or gustatory stimuli were observed by LANDGREN (1957 b).

Certain differences between multimodal responses at the different levels of the path should, however, be noted. Thus thalamic and cortical neurones of this type were sensitive to cooling but rather insensitive to tactile stimulation, whereas the opposite was true of multimodal peripheral "touch fibres". The pattern of tactile response shown by multimodal thalamic and cortical neurones also differed from that of "touch fibres" and unimodal "touch cells". It is therefore unlikely that the central responses would merely be a repetition of the signals from peripheral "touch fibres" which were also discharged by cooling the tongue. To explain the findings a central convergence between tactile and thermal paths has to be assumed, as suggested by LANDGREN (1957 b). Such a convergence may even take place at the medullary level of the path. Multimodal cells were, however, more common in the cortical than in the thalamic population of neurones, which indicates an increasing degree of intermodal convergence at the higher levels of the path.

Although interaction between modalities seems to play a role in the central reception of tongue sensations, it must not be forgotten that the majority of the observed "touch cells" in the thalamic as well as in the cortical population were unimodal. Impulses evoked by tactile stimulation are therefore mainly conducted in specific paths all the way up to the cerebral cortex.

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Thalamic Neurones Responding to Cooling of the Cat's Tongue

By

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Abstract

LANDGREN, S. *Thalamic neurones responding to cooling of the cat's tongue.* Acta physiol. scand. 1960. *48.* 255—267. — In encéphale isolé preparations extracellular spike potentials were recorded from single neurones in the thalamic *nucleus ventralis posteromedialis* (VPM). The effect on these neurones of thermal, mechanical, gustatory and electrical stimulation of the tongue and electrical stimulation of the ipsilateral dorsal ascending trigeminal tract was investigated.

Out of 62 neurones fully investigated 20 responded only to cooling of the tongue, 4 to touch or pressure and to cooling and 1 to warming.

The latency of the response of the "cold cells" was generally 6—14 msec to electrical stimulation of the tongue and 1.0—1.9 msec to stimulation of the tract.

The response of "cold cells" to constant temperatures applied to the tongue and to rapid changes between constant temperature levels was investigated. A drop in temperature caused an increased frequency of discharge, which adapted to a new constant level. In the range 40—20° C the larger the drop in temperature the higher was the initial as well as the maintained frequency. Warming the tongue to 37° C reduced the discharge. Above 45° C an increase was again observed.

The anatomical localization of the "cold cells" was laterally in the medial parvocellular part of VPM.

The response of the appropriate peripheral nerve fibres to cooling and warming of the cat's tongue was described by ZOTTERMAN (1936) and quantitatively analyzed by HENSEL and ZOTTERMAN (1951 a—e), DODT and ZOTTERMAN (1952 a, b) and by DODT (1952, 1953). Thanks to these investigations the peripheral nervous discharge caused by certain defined thermal stimuli is known in great detail. It should therefore be interesting to study responses to thermal

stimuli at the higher levels of the afferent path from the tongue. Such responses were recorded by LANDGREN (1957 a) from single cells in the cortical tongue projection area of the cat. The aim of the present series of experiments was to localize and record from thalamic neurones responding to thermal stimulation of the cat's tongue and to compare their responses with those obtained from peripheral nerve fibres and from cortical neurones.

Methods

The experimental technique has been described in previous publications (*cf.* LANDGREN 1960) and therefore only a brief account of the methods is given here. Cats were used for the experiments. They were anaesthetized with thiopentone sodium (May and Baker), ether, or trilene (ICI). *Encéphale isolé* preparations were made, with local anaesthetic block at C 2. Wounds and pressure points were locally anaesthetized and the general anaesthetic discontinued.

The activity of single neurones in the *nucleus ventralis posteromedialis* of the thalamus was recorded extracellularly with sodiumchloride filled microelectrodes inserted with the aid of a micromanipulator and a stereotactic apparatus. The following stimuli were applied to the tongue: 1) Touch, pressure and stretch, 2) cooling and warming, 3) salty, bitter and acid solutions and distilled water. Cooling and warming were induced by flooding the tongue surface with Ringer's solution or water of known temperature or, when the stability of the recorded action potentials allowed, with a thermode according to the technique of HENSEL, STRÖM and ZOTTERMAN (1951). The effects of electrical stimulation of the tongue surface and of the ipsilateral dorsal ascending trigeminal tract at the mesencephalic level were also investigated.

In order to localize successful recording sites the animal was anaesthetized with thiopentone sodium and intra vital fixation carried out while it was suspended in the stereotactic apparatus and with the microelectrode in position. Ringer's solution and Bodian's solution were used for the perfusion and fixation *in situ*. After fixation the microelectrode was removed and the track localized in histological serial sections, 100 μ thick, celloidin embedded and stained with toluidin blue.

Results

Material

The responses of 116 thalamic neurones were recorded. All were within or in the vicinity of the region of the *nucleus ventralis posteromedialis* (VPM) where electrical stimulation of the tongue surface evokes a negative focal potential with a latency of 3—4 msec. In this population 85 neurones responded to stimulation of the tongue and 62 of them were fully investigated with mechanical, thermal, gustatory and electrical stimulation as described above. Thirty-two of these cells responded only to touch, four only to pressure and four to touch or pressure as well as to cooling of the tongue. Twenty thalamic neurones were only excited by cooling, and one only by warming. Finally one cell was only excited by application of salt solution (0.5 M NaCl) to the tongue.

The neurones responding to tactile stimulation were described in a previous paper (LANDGREN, 1960) and the present report will deal with those influenced by thermal stimuli.

The specificity of the thalamic "cold cells"

The word "specific" may be ambiguous when used in the phrases "specific path", "specific neurone" or "specific response". It implies that the path is private to a certain sensory modality but may also suggest that it is the shortest and fastest path available to that modality for transmission of the evoked signals from the receptors to the cerebral cortex. A "specific neurone" would then be a neurone in the fastest private path of a certain modality and a "specific response" a response of such a neurone. From a practical point of view such a combination may be considered in reasonable agreement with observed facts, but difficulties arise, when paths are found, which are private but not the fastest or in other cases are the shortest without being private. In an attempt to avoid confusion the terms unimodal, bimodal and multimodal are used below to describe neurones responding respectively to one, to two or several types of stimuli. For the sake of convenience neurones responding to cooling are referred to as "cold cells" in analogy with the previously used terms "touch cell" and "touch fibre".

All investigated thalamic neurones were spontaneously active. The frequency of this activity was generally low and irregular and it was therefore as a rule not difficult to decide whether a cell was responding to the applied stimuli or not. One can therefore say definitely that a great majority of the thalamic "cold cells" (20 out of 24) were unimodal, whereas only 4 cells were bimodal. The bimodal "cold cells" responded to touch and cooling or to pressure and cooling of the tongue, but so far no other combinations of effective stimuli have been observed in the thalamic relay nucleus.

The receptive field

The receptive fields of the thalamic "cold cells" were restricted to the tongue. With the present technique the outlines of the field on the tongue were not always easy to define, and they were investigated only in three of the neurones. Two of these had well defined receptive fields on the ipsilateral edge of the tongue which stretched from the level of the canine tooth to that of the first premolar. A drop of Ringer's solution at 20° C or a puff of air within this area was enough to fire a response. The third neurone showed a receptive field on the contralateral half of the tongue.

The response to electrical stimulation

The response of the unimodal thalamic "cold cells" to electrical stimulation of the tongue surface varied from one to a burst of up to 8 spikes (Fig.

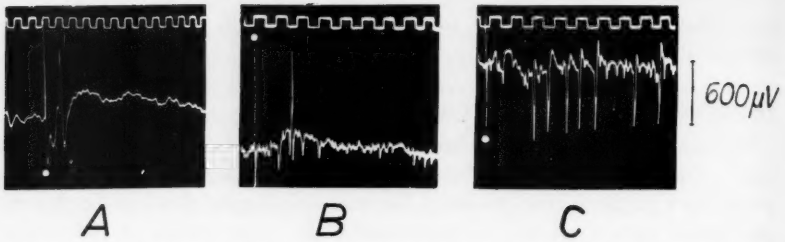

Fig. 1. Records of spikes from thalamic "cold cells" discharged by electrical stimulation of the ipsilateral dorsal ascending trigeminal tract (A) and of the tip of the tongue (B and C). Time calibration A 2 msec, B and C 10 msec. Amplifier time constant: 50 msec. Postitive deflections are recorded upwards.

l B and C). The frequency, averaged over the length of the burst, varied from 50 to 400 per sec in the different neurones.

The latency to the first propagated spike, discharged in a thalamic "cold cell" by electrical stimulation of the tongue surface showed a certain variation, when measured in a consecutive series of sweeps. The range within these latencies varied was less than 2 msec in 4 out of 11 investigated neurones. The maximum range was 17 msec. Most latencies were, however, clustered around a mean value even in the series with a wide range of variation. Part of the variation may be due to the fact that a propagated spike was not always discharged on the first prepotential in the response, and widely divergent values could also be introduced into a series by spontaneously fired spikes.

In the series of latency measurements obtained from each cell the shortest value and the mean were considered of particular interest and the distribution of these values within the population of cells was studied. The shortest latencies ranged from 3.5 to 16.4 msec with a modal value of 7.5 msec. Only 3 of the 11 neurones showed latencies shorter than 6 msec. The mean latencies ranged from 4 to 24 msec (modal value 11 msec) and only two of them fell below 7.5 msec.

The unimodal thalamic "cold cells" thus responded to electrical stimulation of the tongue surface with a latency which was generally 2—3 times longer than that of the corresponding "touch cells" (LANDGREN, 1960). Three "cold cells" did, however, show latencies similar to those found in "touch cells". They were nevertheless very sensitive to cooling of the tongue, whereas no response was evoked by touch or pressure.

The latencies of the bimodal "cold cells" were described in a previous report (LANDGREN 1960). Short as well as long latencies were found. One of these neurones thus showed a latency similar to that of the "touch cells" but from two of them values exceeding 8 msec were recorded.

Four unimodal "cold cells" were tested with electrical stimulation of the

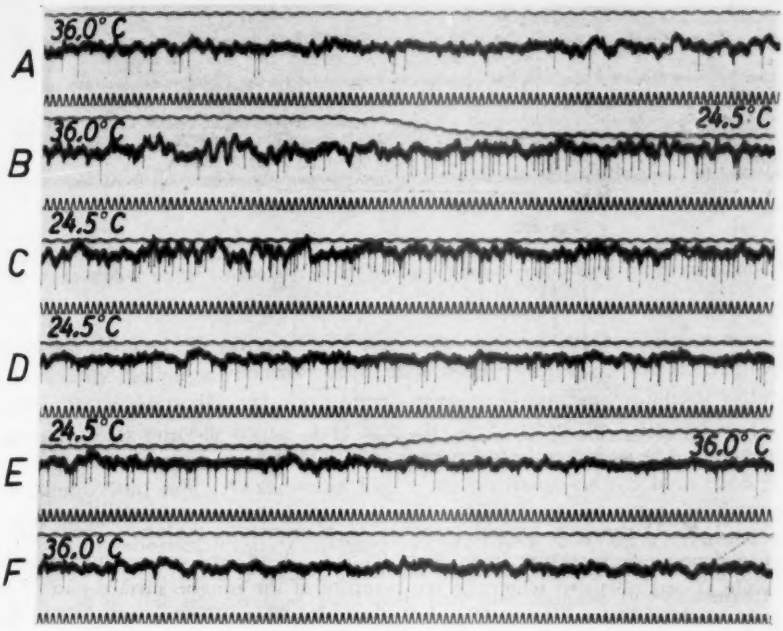

Fig. 2. The response of a thalamic neurone to cooling and warming of the tongue. The temperature of the thermode applied on the tongue surface is recorded by the top beam. 50 cps on lower beam. A—C and D—F are continuous records. The time interval between record C and D was 70 sec. The temperature of the tongue was kept at 24.5° C during this period.

ipsilateral dorsal ascending trigeminal tract, and they all responded to this stimulus. Such a response is seen in Fig. 1 A. In three of the neurones investigated it consisted of one spike only but in one of them the number of spikes varied from 1 to 3. The shortest observed latency of the first spike was 0.9 msec, and the mean latencies ranged from 1.1 to 1.9 msec. These values do not differ from those recorded from thalamic "touch cells".

The response to cooling and warming of the tongue

The thalamic "cold cells" responded to cooling of the tongue with an increase in the frequency of the discharge. Warming of the tongue on the other hand decreased the frequency. Some "cold cells" were very sensitive to cooling of the tongue and responded readily to a drop of cold Ringer's solution or to blowing a puff of air over the tongue surface.

Seven "cold cells" were tested with the thermode technique of HENSEL *et al.* (1951). The effect of sudden changes from one tongue temperature to another was investigated. Changes of approximately 5, 10 and 15° C were applied.

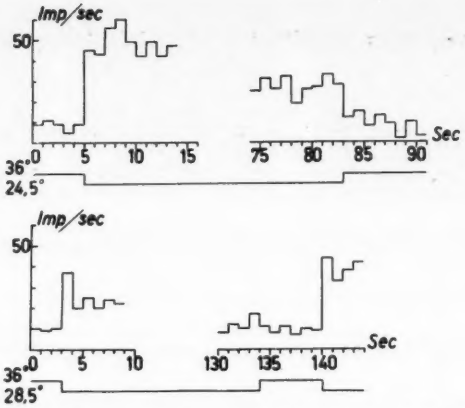

Fig. 3. Histograms showing the impulse frequency of the neurone of Fig. 1 when responding to changes of the tongue's temperature between constant levels.

Fig. 2 A—D shows a typical response of a unimodal thalamic "cold cell" to a drop in temperature from 36 to 24.5° C. A—C are consecutive records while D was obtained when the temperature of the tongue surface had been kept at 24.5° C for 70 sec. E and F show the effect of rewarming. Impulse frequency histograms from this experiment are drawn in Fig. 3. The frequency was counted in 1 sec intervals. A sudden fall in the temperature of the tongue thus caused an immediate increase in the discharge frequency of the thalamic cold cell. The maximum frequency was reached within 4 seconds and then the curve fell to an approximately constant level. In some experiments the temperature was kept constant for a period of up to 4 minutes and the adapted frequency (the steady discharge) of the thalamic neurone remained at a constant level throughout this period. The gradually increasing initial frequency seen in the upper diagram of Fig. 3 was not typical. In most of the neurones investigated the maximum frequency was reached within the first second after the change of the tongue's temperature.

The initial maximum frequency as well as the frequency of the steady discharge increased with increasing intensity of the stimulus *i. e.* with larger drops in the temperature of the tongue, as seen when comparing the lower and upper histogram of Fig. 3. The present material does not allow a quantitative analysis of the steady discharge at different temperatures.

The response of the thalamic "cold cells" to cooling of the tongue was thus very similar to that recorded by HENSEL and ZOTTERMAN (1951 c) from the afferent fibres. The frequency of the steady discharge of the thalamic neurones was, however, higher than that of the peripheral fibres. The steady discharge frequency of the fibres thus never exceeded 10 impulses per sec whereas values

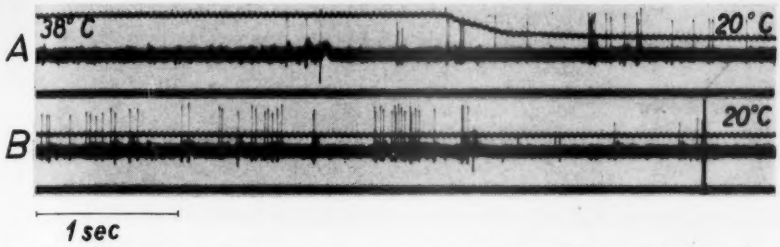

Fig. 4. Phasic response of a thalamic neurone to cooling of the tongue. The temperature of the thermode applied on the tongue is recorded by the top beam. A and B are continuous records.

between 10 and 30 impulses per sec were observed in thalamic neurones. The steady discharge frequency of the thalamic neurones was also less regular than that of the peripheral nerve fibres. Apparently random variations within a range of 10 impulses per sec were generally observed in the thalamus.

A phasic response to cooling of the tongue was observed in one unimodal thalamic "cold cell". As shown in Fig. 4 the discharge evoked by a drop in temperature from 38 to 20° C ends abruptly about 5 seconds after the change to the lower constant level. At a tongue temperature of 20° C the afferent nerve fibres are expected to show a steady discharge, and the observation of a phasic discharge at this temperature level may therefore indicate the existence of thalamic response patterns differing from those seen in the periphery. It can, however, not be excluded that the discharge was cut short by a cold block of the peripheral nerve fibres, which according to DODT (1953) may occur below 22° C.

The decrease in impulse frequency induced by warming of the tongue may be seen in Fig. 2 E and F and in the upper histograms of Fig. 3 and 5. The postexcitatory depression typical for the corresponding responses in the peripheral nerve fibres was less obvious in the records from thalamic "cold cells". A short pause in the discharge lasting about 0.2 seconds, may be seen in Fig. 2 E. Following such a rise in temperature the pause in the discharge of the afferent fibres lasts for seconds (HENSEL and ZOTTERMAN 1951 c and DODT 1952). In many tests a gradual decrease in frequency was seen in stead of a pause and a slow return to a new level of steady discharge. This difference between the peripheral and the thalamic responses is probably due to a desynchronizing effect of the intercalated medullary neurones.

As seen in the top histogram of Fig. 5 the activity of the "cold cell" was reduced to about one impulse per second, when the tongue was flooded with Ringer's solution of 37° C. Similar effects were always observed, when warming the tongue to body temperature. Flooding with water or Ringer's solution of 45° C caused an increase in the frequency of discharge in some of the thalamic "cold cells" (*c. f.* lower histogram of Fig. 5) whereas no effect was seen in others.

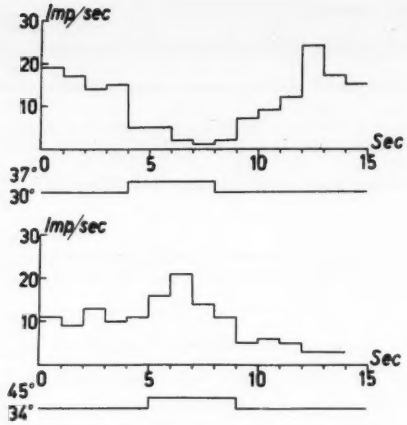

Fig. 5. Impulse frequency histograms showing the response of two thalamic neurones to warming of the tongue.

The increase in activity at temperatures above 45° C was expected as such an effect was demonstrated by DODT and ZOTTERMAN (1952 b) in the "cold fibres" from the tongue.

The response of the bimodal thalamic neurones

The response to touch of the tongue shown by the bimodal thalamic "cold cells" was previously described (LANDGREN 1959). It differed from that of the unimodal thalamic "touch cells" in being less regular and more obviously affected by repetitive stimulation. A tactile response of a bimodal neurone is seen in Fig. 6 A. In this neurone the response appeared in about 50 per cent of the tests. The latency of the burst of spikes was comparatively long but the response was definitely related to the stimulus.

The response to cooling of the tongue shown by these neurones was similar to that of the unimodal "cold cells". As seen in Fig. 6 B a large increase in the activity was caused by a cooling of the tongue from 34 to 15° C. The bimodal neurones were also very sensitive to cooling of the tongue, which is demonstrated by the record D in Fig. 6. Faint puffs of air blown by a ball syringe kept in a thermosflask with Ringer's solution at 20° C were a very effective stimulus. The activity was not due to a mechanical effect since (record E) warmed air blown in stronger puffs onto the tongue surface, evoked no spikes.

The behaviour of these neurones was similar to that shown by some of the bimodal cortical neurones described by LANDGREN (1957 b).

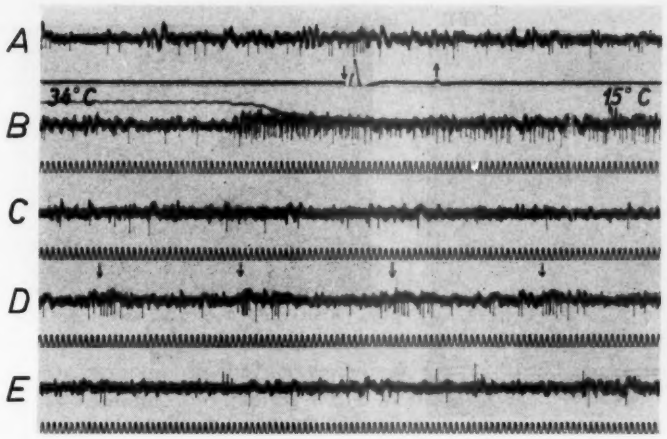

Fig. 6. Records of action potentials from a thalamic neurone responding to touch and cooling of the tongue. A touch between arrows, B cooling from 34 to 15° C, C control showing the impulse discharge when the tongue had adapted to room temperature. D gentle puffs of cool air (20° C), blown onto the tongue with a ball syringe. E warmed air (about 40° C) blown in stronger puffs on the tongue. Note the sensitivity to cooling and long latency of response to touch shown by this neurone. Time 50 cps.

The response of a unimodal "warm cell"

As mentioned above one thalamic neurone was found which only responded to warming of the tongue. It was spontaneously active with a frequency of 3 to 5 impulses per sec at a tongue temperature of about 32° C. Flooding the tongue with Ringer's solution of 42° C increased the frequency to about 20 impulses per sec. Cooling, touch, pressure, spontaneous movements of the tongue or flavoured solutions applied to the tongue were of no effect. This neurone was found close to two unimodal "cold cells" in the same microelectrode track. In two other cases responses to warming of the tongue were observed together with effects of cooling. The activity was, however, due to several units and it was impossible to get clear records of one cell.

The histological localization of the thalamic "cold cells"

The microelectrode tracks along which the thalamic "cold cells" were observed, were localized on histological serial sections. The tracks entered VPM about 1 mm rostrally to the caudal end of the nucleus *i. e.* in the region between its caudal and middle third. They penetrated in a sagittal plane tangential to the lateral border of *nucleus centralis lateralis* and ended laterally in the medial region of VPM which is mainly occupied by small neurones. The course of such a track may be followed on the photographs of a histological section shown in Fig. 7. When the track enters VPM it passes through a region with

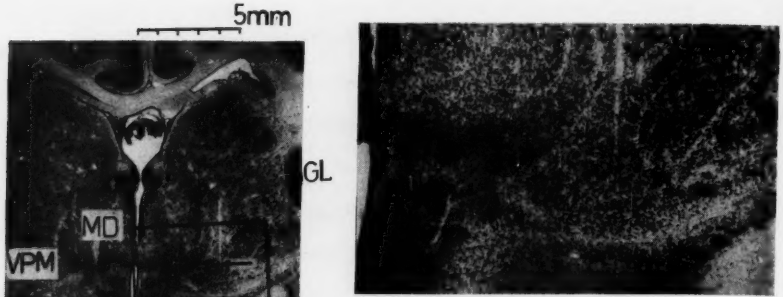

Fig. 7. Microphotographs of a frontal section through the thalamus of a cat showing the localization of a microelectrode track (marked by an arrow in A), at the tip of which records from "cold cells" were obtained. B shows a higher magnification of the framed area in A.
VPM: nucleus ventralis posteromedialis
MD: nucleus medialis dorsalis
GL: corpus geniculatum laterale
100 μ. Toluidine blue.

relatively large neurones before entering into the small cell area. Unimodal "touch cells" and "cold cells" were found along this track, and the microelectrode was left in the position where a "cold cell" was recorded.

The Horsley-Clarke coordinates of the sites of the thalamic "cold cells" were determined by comparing the histological sections with the atlas of JASPER and AJMONE-MARSAN (1954). In the different experiments the value varied within the following range:

A, 8 to 9
L, 3.8 to 4.5
H, 0 to —1

The A and L coordinates are easily obtained with the histological method of localization, but it is difficult to determine the exact position of the microelectrode tip along the dorso-ventral axis, as the very tip was too thin to cause any visible destructions. Micromanipulator readings of the H coordinates related to measurements on the sectioned brain were in agreement with the histological localization.

The localization of the region in the mesencephalon, where the dorsal ascending trigeminal tract was stimulated was previously described by APPELBERG, KITCHELL and LANDGREN (1959).

Discussion

It is of great interest that the majority of the thalamic "cold cells", in the population investigated, did not respond to any other physiological stimulus than cooling of the tongue. Unimodal "cold cells" were also found in the cortial tongue projection area by LANDGREN (1937 a). These observations demonstrate, that impulses evoked by cooling of the cat's tongue are conducted right up to the cortical level in paths inaccessible to other types of stimuli, at least under the present experimental conditions. The findings support the idea that specific receptors and specific afferent paths exist also for the skin modalities of sensation. This idea was doubted by SINCLAIR (1955). One of the most important objections against the theory was that critical histological investigations by WEDDELL, PALLIE and PALMER (1954) failed to show a differentiated organization of the nerve endings in the skin. However, results obtained by recording the impulse traffic in single afferent nerve fibres from the skin and the tongue of the cat demonstrate the existence of nerve endings with a high degree of functional specificity. A brief review of these results may be of interest.

Generally mechanoceptive fibres from the cat's tongue were not excited by thermal stimuli when the intensity of these stimuli was kept within physiological limits. This statement is not seriously invalidated by the observation that *some* "touch fibres" from the tongue also responded to cooling of the receptor layer (*cf.* HENSEL and ZOTTERMAN 1951 e, and DODT 1953). Mechanoreceptors with high specific sensitivity to mechanical stimuli were also found by BOMAN (1959) in the infraorbital region of the cat's skin.

According to HENSEL and ZOTTERMAN (1951 c) the "cold fibres" from the tongue did not respond to mechanical stimuli. They did, however, respond to warming, but were easily differentiated from the "warm fibres" of the tongue (*cf.* DODT and ZOTTERMAN 1952 a), because the frequency of the "cold fibre" discharge was reduced to zero at a temperature of the tongue between 40 and 45° C and only increased again at temperatures between 45 and 50° C (DODT and ZOTTERMAN 1952 b). The "warm fibres" on the other hand showed a maximal steady discharge frequency, when the temperature of the tongue was about 40° C. The "warm fibres" were also excited by cooling, but this response was phasic in nature and a rather intense cooling of the tongue was required to elicit it. "Cold fibres" on the other hand showed a steady discharge between 40 and 15° C the frequency of which was very sensitive to changes in the temperature.

The thermoceptive group A fibres from the cat's skin seems to differ from those of the tongue. Thus WITT (1959) found that a large group of thermoceptive skin fibres were also sensitive to mechanical stimuli. C-fibres responding with great sentitivity to warming of the skin but highly insensitive to mecanical stimuli were, however, recently observed by HENSEL, IGGO and WITT (personal communication).

In spite of the limitations mentioned above it seems clear, that receptors with a high degree of specificity to mechanical stimuli are common in the mucous membrane of the tongue and in the skin of the cat. Receptors responding to cooling or warming with a relatively high degree of specificity are further found in the cat's tongue. These conclusions are compatible with the present observations of unimodal thalamic neurones.

Another interesting finding made in the present series of experiments was that thalamic neurones responding to cooling of the tongue were fired by electrical stimulation just laterally to *fasciculus longitudinalis medialis* in the mesencephalon. According to histological investigations several paths containing fibres from the medullary nuclei to the thalamus are found in this region (see HASSLER 1948). One of these paths is the dorsal ascending trigeminal tract, which consists of uncrossed fibres from the chief sensory trigeminal nucleus to VPM. Using Marchi's technique CARPENTER (1957) has recently studied the course of this tract in the monkey and the localization of our stimulating electrodes agrees very well with his localization of the dorsal ascending trigeminal tract at the mesencephalic level.

As shown in a previous report (LANDGREN 1960), thalamic neurones responding to tactile stimulation of the tongue were also excited by electrical stimulation in the region of the dorsal ascending trigeminal tract. The observations suggest, that ipsilateral mechanoceptive and thermoceptive paths from the tongue travel together in this tract. The conduction velocity of the peripheral "touch fibres" from the tongue is about three times as large as that of the peripheral "cold fibres". This difference in conduction velocity apparently does not exist in the dorsal ascending trigeminal tract, as thalamic "touch cells" and "cold cells" responded with the same latency, when fired by electrical stimulation of this tract.

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The Association between the Relaxing and the Lactic-Acid Stimulating Effects of Adrenaline in Smooth Muscle

By

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Abstract

MOHME-LUNDHOLM, E. *The association between the relaxing and the lactic-acid stimulating effects of adrenaline in smooth muscle.* Acta physiol. scand. 1960. *48.* 268—275. — According to FURCHGOTT (1955) the relaxing effect of adrenaline on rabbit intestine even emerged under conditions in which the muscle probably was incapable of producing lactic acid. On duplicating this experimental condition and determining glycogenolysis and lactic acid production, the author found that adrenaline's relaxing effect on smooth muscle was still associated with stimulation of the lactic acid production.

In earlier papers data were presented which strongly suggested that the relaxing effect of adrenaline on smooth muscle was linked with its stimulation of the lactic acid production and glycogenolysis (MOHME-LUNDHOLM 1953, 1956 a, b, 1957, LUNDHOLM and MOHME-LUNDHOLM 1957). This theory was criticized by FURCHGOTT (1955), who had found that rabbit gut stored in glucose-free salt solution gradually lost its activity; after approximately two hours the magnitude of the contractions had decreased to 10—20 per cent of the initial values (FURCHGOTT and SHORR, 1946, FURCHGOTT and WALES 1951, 1952). When a nutrient such as pyruvic acid or acetate was administered to such substrate-depleted gut, it stimulated the latter's activity and tone. Adrenaline, however, had a distinct relaxing effect; and as the gut, according to FURCHGOTT, had exhausted its carbohydrate reserves this effect could not, he thought, be due to stimulation of the lactic acid production. FURCHGOTT found also that glyceraldehyde in a concentration of 0.01 M inhibited almost totally the stimulating action of glucose on substrate-depleted gut, whereas the relaxing effect of adrenaline was not depressed. This observation too, militated against the assumption of an association between the relaxing effect and the stimulation.

Since FURCHGOTT, however, had not determined the lactic acid production under these experimental conditions, his arguments were based only on indirect evidence. I decided, therefore, to ascertain whether that production was in fact inhibited under FURCHGOTT's experimental conditions.

Methods

The experiments were performed on rabbit intestine. Immediately after killing of the animal by means of air emboli, and rinsing of the intestine in Tyrode solution, ten preparations, each weighing approximately 0.5 g, were taken from a single area of the jejunum. Two of them (control samples) were immediately assayed for lactic acid and glycogen (MOHME-LUNDHOLM 1953, LUNDHOLM and MOHME-LUNDHOLM 1957). The remaining eight were mounted in organ baths containing 20 ml Tyrode solution at 38°C. Tone and activity were registered with an isotonic recorder having a load of 2 g. On completion of the experiment both the preparations and their Tyrode solutions were subjected to lactic acid assay. Changes in lactic acid production were calculated from the difference between the totalled contents in preparations and solution at the end of the experiment and the content in the control samples. Glycogenolysis was computed from the changes in glycogen content of the preparations.

Glyceraldehyde, due to a chemical similarity with lactic acid, reacted in some degree as lactic acid when assayed *ad modum* FRIEDEMANN and GRAESER (1933). With this procedure it was equivalent to approximately one-hundredth the amount of lactic acid. When determining the lactic acid content of Tyrode solution containing glyceraldehyde, it was therefore necessary to analyze control solutions having the same glyceraldehyde content as the former, in order to calculate the actual lactic acid production.

Results

Lactic Acid Production and Glycogen Content of Substrate-Depleted Intestine

The first question was whether the carbohydrate reserves of rabbit gut were in fact exhausted, as Furchgott had assumed, after only two hours or so in glucose-free Tyrode solution. Under the conditions of my experiments the activity of the gut was generally very good after two hours in such solution; not until 5—6 hours had elapsed was it, as a rule, totally abolished. Indeed, in a few experiments approximately 10 per cent of the maximum activity was still present after that time. In the first run of experiments wherein the anaerobic lactic acid production and the glycogenolysis were determined in substrate-depleted preparations, eight preparations were mounted in glucose-free Tyrode solution, aerated with carbogen (93.5 % O_2 + 6.5 % CO_2), and the activity recorded for 6 hours. Following this procedure four preparations and their Tyrode solutions were taken for lactic-acid and glycogen assay. The Tyrode solutions of the remaining four were changed after 6 hours and the fresh solutions aerated with nitrogen, enabling the lactic acid production and glycogenolysis to be studied under anaerobic conditions. These preparations and their Tyrode solutions were assayed after a further 2 hours.

The results are presented in Table I, from which it is evident that during the 6 hours of aerobic conditions the preparations oxidized some of their

Table I. Anaerobic lactic acid production and glycogenolysis in substrate-depleted rabbit intestine.

The gut was immersed first for six hours in glucose-free Tyrode solution in O_2, and then for two hours in N_2. The control samples had a mean lactic acid content of 72.7 mg per cent and a glycogen content of 87.0 mg per cent.

Test no.	Lact. acid prod. mg/100 g		Glycogenolysis mg/100 g	
	6 h in O_2	2 h in N_2	6 h in O_2	2 h in N_2
1	− 38.0	37.9	31.5	10.2
2	1.5	39.0	17.4	27.1
3	− 29.9	25.0	21.4	17.7
4	− 21.1	4.9	—	7.0
5	− 38.4	22.8	59.3	60.2
6	− 78.5	33.4	7.1	19.2
7	− 73.2	35.5	5.3	21.0
Mean	− 39.7 ± 10.6	28.4 ± 4.6	23.7 ± 8.1	23.2 ± 6.7
P	< 0.01	< 0.001	< 0.05	< 0.05

original lactic acid content. It is not impossible that a certain amount of bacterial oxidation contributed to the lactic acid depletion. The glycogenolysis averaged 23.7 mg per 100 g tissue. During the two hours under anaerobic conditions the substrate-depleted preparations produced an average of 28.4 mg lactic acid per 100 g; the glycogenolysis was almost as great — 23.2 mg per 100 g. Even after two hours in N_2 the preparations still had a glycogen content averaging 40.1 mg per cent. These results suggest that it is not possible to exhaust entirely the carbohydrate reserves of smooth muscle.

Effects of Adrenaline on the Carbohydrate Metabolism of Substrate-Depleted Rabbit Intestine

In the next run of experiments I sought to ascertain whether adrenaline stimulated the glycogenolysis and lactic acid production of substrate-depleted muscle. Following glycogen and lactic acid assay of two control specimens, eight preparations were mounted in organ baths, which were aerated with carbogen throughout the experiment. After 6 hours two preparations were taken for lactic acid assay, and the Tyrode solutions of the other six were changed. To three of these six, adrenaline was added in a dose of 20 μg per 20 ml with 15-minute intervals. All preparations and their solutions were taken after 60 min for glycogen and lactic acid assay. Any tone or activity remaining in the preparations was inhibited by adrenaline. The results are shown in Table II. Where the Tyrode solution was changed after 6 hours the lactic acid production for the next hour averaged 3.0 ± 1.34 mg per 100 g, whereas in corresponding experiments with adrenaline the average was 6.2 ± 1.70 mg per 100 g. Adrenaline stimulated the lactic acid production by an average of

Table II. Effect of adrenaline on lactic acid production and glycogenolysis of substrate-depleted rabbit gut. Incubation time: 1 hour. The gut previously mounted for 6 hours in an organ bath containing glucose-free Tyrode solution.

Test no.	Control		Adrenaline	
	Lactic acid prod. mg/100 g	Glycogenolysis mg/100 g	Increase of lact. acid prod. over control mg/100 g	Increase of glycogenolysis over control mg/100 g
1	4.3	5.5	5.1	0.9
2	5.2	17.1	4.6	5.6
3	4.8	4.2	2.8	1.0
4	− 4.8	7.7	4.2	4.7
5	4.6	7.0	— 1.8	0.5
6	3.9	14.0	4.2	9.4
Mean	3.0 ± 1.34	9.3 ± 2.09	3.18 ± 1.04	3.68 ± 1.40
P	< 0.1	< 0.01	< 0.05	< 0.05

3.18 ± 1.04 mg per 100 g, or more than 100 per cent. The increase was statistically probable. Similar results were obtained in the case of glycogen. For the period from 6 to 7 hours without adrenaline, the glycogenolysis amounted to 9.3 ± 2.09 mg per 100 g; but following addition of adrenaline it rose to 12.9 ± 3.35 mg per 100 g, or by 3.68 ± 1.40 mg per 100 g. With increased lactic acid production there was commensurate breakdown of glycogen. The magnitude of adrenaline's lactic acid stimulating effect was approximately 50 per cent of the lactic acid production under anaerobic conditions.

Earlier experiments on preparations having full activity and major carbohydrate reserves had shown that adrenaline possessed a glycogenolytic action only in the absence of glucose, but even then the glycogen loss was too small to account in full for the lactic acid production (LUNDHOLM and MOHME-LUNDHOLM 1957). As is evident from these later experiments, however, the glycogen content was comparatively little affected upon depletion of the carbohydrate reserves, and the magnitude of carbohydrate breakdown was probably governed, in due course, by the rate of glycogenolysis. There seems little possibility of totally divesting smooth muscle of its glycogen, for the particular enzyme phosphorylase which activates the reaction, glycogen $+ H_3PO_4 \underset{\longleftarrow}{\overset{\text{phosphorylase}}{\longrightarrow}}$ glucose-1-phosphate, is itself inactivated with advanced substrate depletion (CORI 1956). Since, however, adrenaline activates phosphorylase (CORI and SUTHERLAND 1951, SUTHERLAND 1951, 1952), it would scarcely be possible to induce such glycogen loss as to preclude a glycogenolytic effect of adrenaline.

Table III. Effect of 0.01 M DL-glyceraldehyde (GA) upon the anaerobic lactic acid production of rabbit gut from glucose as well as from glycogen and intermediate carbohydrates. The lactic acid production was determined for a two-hour period.

Test no.	Tyrode 0.1 % glucose		Tyrode without glucose	
	Control mg/100 g	0.01 M GA mg/100 g	Control mg/100 g	0.01 M GA mg/100 g
1	1,026.9	746.0	71.9	254.4
2	510.6	211.2	95.5	158.5
3	598.4	248.0	275.5	322.1
4	1,272.3	563.4	205.7	218.3
Differences:	− 409.9 ± 100.7		+ 76.2 ± 36.9	
P	< 0.05		< 0.2	

Action of DL-Glyceraldehyde on the Carbohydrate Metabolism of Rabbit Intestine

This action is complex, for glyceraldehyde depresses the glucose metabolism in a much lower concentration than that required for inhibition of glycogenolysis. STICKLAND (1941) in experiments on muscle extracts, found that DL-glyceraldehyde in a concentration of 0.003 M reduced the lactic acid production from glucose by 85—95 per cent, whereas 0.05 M reduced the corresponding production from polysaccharides by only 5—30 per cent. LARDY, WIEBELHAUS and MANN (1950) reported that inhibition of the glucose metabolism was entirely attributable to L-glyceraldehyde, which inhibited hexokinase. NEEDHAM, SIMONOWITCH and RAPKINE (1950) demonstrated that D-glyceraldehyde in high concentrations depressed the lactic acid production by competitive inhibition of the oxidation of D-glyceraldehyde phosphate by glyceraldehyde phosphate dehydrogenase.

The concentration of glyceraldehyde which FURCHGOTT (1955) employed to inhibit the glucose metabolism in rabbit intestine was approximately eight times lower than that required for depressing the lactic acid stimulating and the relaxing effects of adrenaline in experiments on bovine tracheal muscle (MOHME-LUNDHOLM 1953). The failure of glyceraldehyde to inhibit adrenaline's relaxing effect could well be explained, therefore, by the supposition that the concentration used by FURCHGOTT merely depressed the glucose metabolism without affecting the glycogen metabolism.

In order to demonstrate this selective effect of glyceraldehyde on the glucose metabolism of rabbit gut I took eight preparations, as before, and mounted them in organ baths. Four of these contained Tyrode solution with 0.1 per cent glucose; the others glucose-free Tyrode solution. To three preparations in each group was added 0.01 M DL-glyceraldehyde solution (18 mg/20 ml), after which the solutions were aerated with N_2. After 2 hours the preparations and

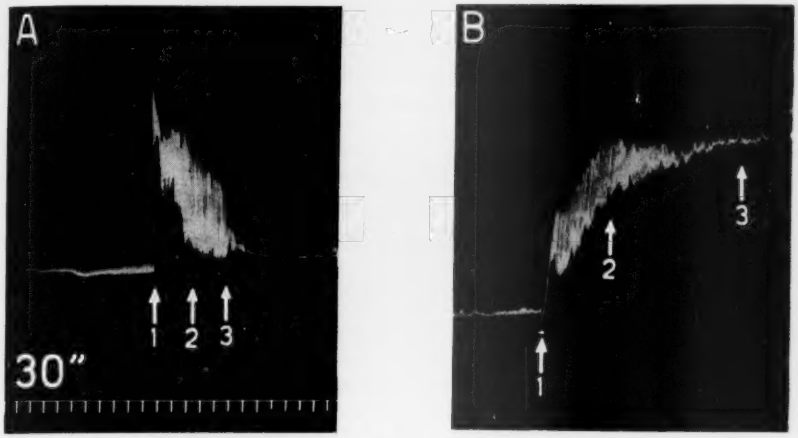

Fig. 1. Rabbit gut in Tyrode solution containing 0.1 per cent glucose.

A. At arrow 1, carbacholine $2.5 \cdot 10^{-7}$; at arrow 2, adrenaline $2.5 \cdot 10^{-7}$; at arrow 3, adrenaline $1 \cdot 10^{-6}$.

B. The Tyrode solution contained 0.04 M DL-glyceraldehyde. At arrow 1, carbacholine $2.5 \cdot 10^{-7}$; at arrow 2, adrenaline $2.5 \cdot 10^{-7}$; at arrow 3, adrenaline $5 \cdot 10^{-6}$.

The inhibition of pendular movements in B is unlikely to have been caused by adrenaline, since at lower concentrations of glyceraldehyde, adrenaline had a tone-reducing effect but did not influence those movements.

their Tyrode solutions were assayed for lactic acid. — The anaerobic activity of the gut was far greater in the experiments with than in those without glucose. Glyceraldehyde inhibited this stimulating effect of glucose on the activity. — The results are summarized in Table III. The mean lactic acid content in four such experiments without addition of glucose or glyceraldehyde had increased by 162.2 mg per 100 g. After glyceraldehyde the lactic acid production had increased to 238.1 mg; yet it inhibited to a large extent the corresponding stimulation produced by glucose. This experiment accordingly demonstrated that glyceraldehyde in the concentration used by FURCHGOTT inhibited only the glucose metabolism and not the glycogen metabolism of rabbit intestine. Since adrenaline, as shown previously, has a glycogenolytic effect on rabbit gut in the absence of glucose, it is unlikely that its lactic acid stimulating effect was inhibited in the experiments reported by FURCHGOTT.

Influence of DL-Glyceraldehyde on the Relaxing Effect of Adrenaline Upon Rabbit Intestine

Glyceraldehyde in a concentration of 0.01 M did not influence the relaxing effect of adrenaline on rabbit gut under aerobic conditions; nor did it affect the spontaneous activity. The latter was depressed when the glyceraldehyde

concentration was increased to 0.04—0.08 M, but carbaminoylcholine still stimulated the tone and pendular movements. At this concentration the relaxing effect of adrenaline was almost totally blocked (Fig. 1), as it had been in previous experiments on bovine tracheal muscle.

Discussion

The findings reported above show that under the conditions described by FURCHGOTT the lactic acid production of rabbit intestine, contrary to his assumption, had not ceased.

Certain data reported in recent years lend indirect support to the view that the relaxing effect of adrenaline is secondary to its action on the lactic acid production. BÜLBRING (1953) in experiments on guinea-pig taenia coli, found that the former effect had little or no influence on the oxygen consumption of the muscle, thus confirming previous observations by RAO & SINGH (1940), among others.

BÜLBRING (1957), using intracellular micromethods in subsequent experiments, noted that adrenaline caused hyperpolarization of the smooth-muscle cell. An increase of the membrane potential, she observed, was regularly associated with relaxation. But hyperpolarization of the cell membrane must imply either that the concentration of negative ions has increased in the cell or that the positive ions have decreased. On determining the potassium metabolism of the smooth-muscle cell BORN and BÜLBRING (1956) found, however, that adrenaline did not increase the potassium release from taenia coli but instead accelerated its uptake of potassium. Hence the potassium metabolism affords no explanation of hyperpolarization following adrenaline. FENN and COBB (1935), experimenting on frog muscle, recorded an increased potassium uptake when the acidity was raised by lactic acid or carbon dioxide. The effect of adrenaline on the membrane potential and the potassium metabolism may, therefore, be secondary to the increase in the intracellular content of lactic acid.

BÜLBRING (1957) found that adrenaline abolished the action potentials in taenia coli. However, the influence of drugs on the activity of smooth muscle appears at least partly to be independent of their action on the membrane potentials, for EVANS, SCHILD and THESLEFF (1958) were able to produce contraction as well as relaxation in completely depolarized smooth muscle. In glycerine extracted skeletal muscle fibres GOODALL and SZENT-GYÖRGYI (1953) and LORAND (1953) found that a pH shift from 7.2 to 6.2 was able to reversibly abolish the tension produced by adenosinetriphosphate in the presence of phosphocreatine and one of the "relaxing factors" namely adenosinetriphosphate-phosphocreatine transphosphorylase. The possibility that an increase of lactic acid in the muscle cell may influence the contractile proteins by activating a relaxing factor must thus be considered.

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Correlation between the Psychological Significance of Stimuli and the Magnitudes of the Startle Blink and Evoked EEG Potentials in Man[1]

By

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Abstract

LARSSON, L.-E. *Correlation between the psychological significance of stimuli and the magnitude of the startle blink and evoked EEG potentials in man.* Acta physiol. scand. 1960. *48*. 276—294. — The effect of variations in the psychological significance of stimuli on evoked potentials in the EEG and on the startle blink has been studied in man. Three different experimental conditions were set up under which the stimuli were assumed to have a high, a moderate and a low significance, respectively. It was found that the magnitudes of the startle blink and the non-specific EEG response were directly correlated to the degree of significance thus defined. The differences between the responses under the three experimental conditions were statistically significant. The short-latency response to peripheral nerve stimulation recorded from the contralateral somatosensory region was more resistant. The cardiac and respiratory rates as well as the EEG from the posterior scalp regions were also recorded under the three experimental conditions. It was found, among other things, that under the condition in which the subjects tensely waited for a stimulus and accordingly must have been highly alert the EEG contained a large amount of alpha waves in contrast to the desynchronization of the record which occurs under other conditions of wide-awakeness.

It is well known that stimuli that have the same physical character and intensity may differ greatly in their power to attract attention, depending upon the significance of the stimulus for the individual at the time it is delivered. A correlation between the psychological significance of stimuli and EEG

[1] Presented at the meeting of the Scandinavian EEG Society held in Gothenburg in September 1958.

responses, particularly alpha blocking, has been observed by several workers (*e. g.* LOOMIS, HARVEY and HOBARTH 1936, BAGCHI 1937, LI, JASPER and HENDERSON 1952, JASPER, AJMONE-MARSAN and STOLL 1952, BREMER and TERZUOLO 1954). More recently HERNÁNDEZ-PEÓN *et al.* (1955 a, c, 1956 a, 1957 b, c) showed in the cat that evoked potentials in sensory pathways at different levels of the central nervous system are abolished during habituation and distraction of attention. They concluded (1957 c) that the "significance of the stimulus must be considered the adequate correlate not only for behavioural habituation but also for 'afferent neuronal habituation' as a physiological fact". ZISLINE and NOVIKOVA (1955) observed in man that the response to flickering light is increased in amplitude if the subject counts the flashes, an effect that may be correlated to an increase in the significance of the stimuli when they are counted. The observations of HERNÁNDEZ-PEÓN and DONOSO (1957 a) are also of great interest in this connection. These workers recorded photically evoked potentials from electrodes deeply implanted in the occipital lobe of four subjects. They found that the evoked potentials were reduced or abolished when the subject was given an arithmetical problem. In one highly suggestible subject the magnitude of the potentials paralleled the perceived intensity of the light according to previous verbal suggestions. Similar observations were reported by JOUVET and COURJON (1958).

The purpose of the present study was to investigate in man the relationship between the psychological significance of otherwise identical stimuli, on the one hand, and the magnitudes of the startle blink (STRAUSS 1928, LANDIS and HUNT 1939) and the non-specific EEG response (DAVIS 1939), on the other. The short-latency response in the somatosensory area to peripheral nerve stimulation described by DAWSON (1947) was also studied in this respect.

Distinct experimental conditions were set up under which the stimuli were presumed to possess a definite degree of significance.

In order to obtain additional information regarding these experimental conditions the respiratory and cardiac rates and the EEG from the posterior scalp regions were recorded simultaneously in some of the subjects.

Material and Methods

The investigations were carried out on 17 waking adult subjects. None of them were known to be suffering from any organic disease of the central nervous system. Throughout the experiments the subjects sat comfortably in an armchair with their eyes open.

Recording Technique

The electrodes consisted of chlorided silver discs attached to the skin with collodion. The non-specific EEG response was recorded from a pair of electrodes on the vertex, one in the midline immediately in front of a line between the two mastoid processes and

one 3 to 4 cm further back[1]. With this electrode placement the non-specific response is generally recorded as a biphasic wave with the initial phase deflected upward and with a latency of about 50 msec. As a rule it is possible to show that this initial phase is surface negative (DAVIS 1939).

In several experiments recordings were also made from the scalp region corresponding to the somatic sensory projection area. When the contralateral median nerve is stimulated at the wrist the response from this area consists of several waves the first of which is surface positive and has a latency of about 20 msec (DAWSON 1947, LARSSON 1953). In the illustrative figures this initial component has an upward deflection. After further one wave of similar appearance but, often, of longer duration, the response more or less assumes the shape of the non-specific response. No attempt was made to determine the polarity of the individual components in the different responses in each individual case since it was considered unnecessary for the present problem.

The startle blink was recorded from a pair of electrodes placed over the orbicularis oculi muscle.

The recordings were made with conventional condenser-coupled amplifiers connected to a double-beam cathode-ray oscilloscope. The time constant was 0.1 sec. In most of the experiments the photographic superimposition technique (DAWSON 1947) was used with ten cathode-ray sweeps for each picture. While this technique has many advantages, it does not always permit accurate measurements. Therefore in some experiments the amplitude of each response was measured separately and the values treated statistically.

When measuring the individual EEG responses it must be borne in mind that they are necessarily mixed with the "spontaneous" activity which is often of the same order of magnitude as the response. The amplitude of the responses as well as the latency to their maximum and minimum points may therefore seem to undergo large variations (see Fig. 1 A). Thus, the true maximum and minimum points and therewith the amplitude are difficult to determine. The measurement of the responses in a given experiment was therefore done in two stages. First, the photographic tracing of each response was projected in turn onto paper with the aid of an enlarger and drawn off, thus providing a superimposed response (Fig. 1 B). With the aid of this drawing the mean maximum and minimum points of the responses were estimated. Three perpendicular lines (t—u, v—x, y—z) were drawn at the beginning and through the maximum and minimum points (Fig. 1 B). In the second stage the record of each single response was again projected, but now shifted parallel to the superimposed drawing (see Fig. 1 C). The amplitude of each response was then obtained by measuring the difference in level between the points at which the lines v—x and y—z cut through the tracing. The method requires that the latency of the responses remains unaltered under the different experimental conditions. Should the latency be altered, a separate superimposition drawing must be done for each experimental condition. In the present investigation this was unnecessary.

Measurement of the individual blink responses was done by projecting the record onto paper with the enlarger. The area covered by the response was then measured

[1] In a recently published paper OSWALD (1959) suggests that the non-specific EEG response may be an artefact caused by a backward twitch of the ears and scalp. Since such twitches are seen only in rare instances as part of very strong startle reactions (LANDIS and HUNT 1939, p. 22) it is highly improbable that they play an important part in the production of the non-specific EEG response. Stimuli of the strength used in the present investigation do not evoke reactions stronger than a blink of the eyelids. Moreover, it has been found that stimuli of considerably greater intensity fail to cause a twitch of the scalp, as tested with the aid of strain gauges (LARSSON 1960).

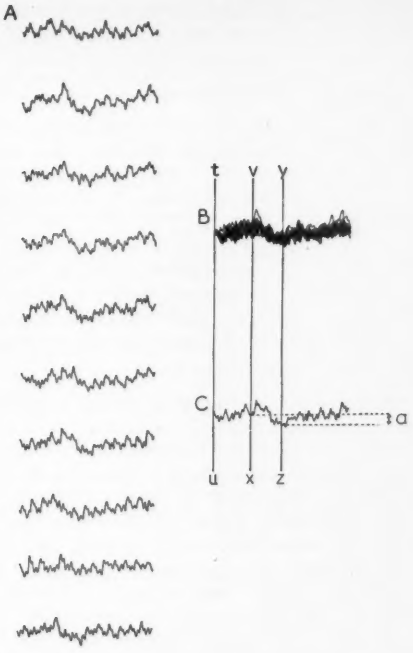

Fig. 1. Method for measurement of single non-specific EEG responses. (In this case recorded from the vertex following auditory stimuli.) The stimulus started the sweep. *A* illustrates the variability of individual responses. *B*, superimposed drawing of the individual responses in *A*. *C* illustrates determination of the amplitude of the individual response based on estimation of the average maximum and minimum in *B*.

with a planimeter. On account of the technique used the magnitudes of both the EEG responses and the startle blink are expressed in arbitrary units.

The EEG and the respiratory and cardiac rates were recorded with a Grass Model III-A EEG instrument.

Respiratory rate. The transducer consisted of a rubber tube about 3 cm in length filled with a solution of $CuSO_4$, which was applied over the subject's chest and held in position with a belt. The respiratory movements stretched the tube and altered the resistance between its two ends. The variations in resistance were recorded on the EEG instrument through a simple electric circuit. The duration of four respirations was measured at given intervals. The values obtained were treated statistically and the mean value was converted into frequency units.

Cardiac rate. The ECG was recorded from the chest. The duration of 15 heart beats was measured and the values obtained were treated as above.

EEG. The EEG was recorded from the posterior midline regions. First, the maximum amplitude of the alpha waves was estimated. Then all the alpha waves with an amplitude exceeding half the maximum amplitude occurring over a 20-second period of recording were counted.

The determination of the respiratory and cardiac rates and the counting of the alpha waves was always done during the same period of recording.

Stimulation Technique

The peripheral nerve stimulus consisted of an electric shock to the left median nerve at the wrist. The shocks were of 1 msec duration. The auditory stimulus consisted of

Table I. Order of experimental conditions in the six experiments which form the basis of the statistical analysis

Subject	Order of experimental conditions					
S-ng	M	L	H	M	L	H
K-n	M	L	H	M	L	H
S-n.....	L	H	M	L	H	M
F-i.....	L	H	M	L	H	M
L-t.....	H	M	L	H	M	L
J-n.....	H	M	L	H	M	L

H = reaction-speed condition (High significance)
M = relaxation (Moderate significance)
L = auto-stimulation condition (Low significance)

a click in a pair of earphones connected to the stimulator unit. The stimuli were given at irregular intervals of 30 to 90 sec.

In preliminary experiments several methods were used to influence the psychological significance of the stimulus for the subject. Methods which it was assumed would increase the significance included questioning the subject regarding the nature of the stimulus or asking him to compare the intensity of one stimulus with another. Methods which it was assumed would diminish the significance of the stimulus were distraction of the subject's attention by conversation or the assignment of simple tasks before the stimulation. About one half of the subjects, however, were investigated under three distinct experimental conditions as follows.

Reaction speed (Stimuli of high (H) significance). The subject was informed that the object of the experiment was to test his speed of reaction. He was provided with a switch which he could easily depress. In front of him was placed a lamp. He was given no instructions other than to press the switch as soon as he perceived the stimulus. The current reached the lamp 200—300 msec after the application of the stimulus. Failure to break the circuit within this period resulted in the lighting of the lamp. The subject was told that lighting of the lamp denoted a poor reaction speed; it was assumed that this would stimulate him to wait intently for the next stimulus. That this assumption was correct was confirmed by the subject's own opinion and the fact that frequently a subject would press the switch on receiving stimuli other than those to which he was instructed to react. The latter is behavioural evidence that the subject paid more than usual attention not only to the stimuli tested but also to other stimuli.

Relaxation (Stimuli of moderate (M) significance). The subject was asked to relax and instructed to ignore the stimuli.

Auto-stimulation (Stimuli of low (L) significance). The subject was given a stop-watch together with a list of the times when the stimulus was to be released. He was instructed to keep strictly to the times stated. This necessitated concentration of his attention on the hand of the stop-watch, a concentration which it was considered would be especially increased at the moment the hand reached the point at which the stimulus was to be released. It was assumed that this would distract his attention from the stimulus itself.

Statistical Analysis

For a statistical check of the results six of the subjects underwent a special investigation. They were given a series of 10 to 11 click stimuli under each of the three experi-

Table II. The magnitude of the non-specific EEG response to stimuli of different psychological significance

Mean values ± standard errors[1]

Subject	Experimental Conditions			Individual average
	Reaction-speed High significance	Relaxation Moderate significance	Auto-stimulation Low significance	
S-ng	12.8 ± 1.8	9.5 ± 2.0	2.4 ± 1.7	8.2
K-n	9.7 ± 2.0	6.1 ± 2.1	2.5 ± 2.0	6.1
S-n	18.2 ± 1.3	9.7 ± 1.2	1.4 ± 1.3	9.8
F-i	17.6 ± 2.2	11.3 ± 2.1	0.5 ± 2.1	9.8
L-t	11.6 ± 1.8	3.9 ± 1.8	1.2 ± 1.8	5.6
J-n	2.9 ± 1.1	1.6 ± 1.1	−0.4 ± 1.1	1.4
Average	12.1 ± 1.2	7.0 ± 1.2	1.3 ± 1.2	6.8

Analysis of variance of mean values

Source of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio	Statistical significance
Between individuals	5	154.163	30.83	3.75	*
Between experimental situations	2	354.655	177.33	21.57	***
Residual	10	82.232	8.23	—	—
Total	17	591.050	—	—	—

[1] The standard errors of the mean values presented in the main body of tables II through VI have been computed from individual measurements. In computing the standard errors of the averages at the bottom of the table the residual variance obtained from the analysis of variance has been used.

mental conditions. When the three series were completed they were repeated once in the same order. The order of the experimental conditions varied from subject to subject (see Table I).

In the statistical analysis of the data obtained the individual measurements were first studied. On each individual there were $2 \times (10 \text{ to } 11) = 20$ to 22 such measurements for each experimental condition. The mean values and the standard errors for these groups of values are presented in the main body of Table II to VI.

The $3 \times 6 = 18$ mean values were then subjected to significance analysis by means of analysis of variance. The results are presented at the bottom of Table II to V.

As regards computative methods used the reader is referred to standard statistical textbooks. The following four degrees of significance have been used:

$P > 0.05$	not significant
$0.05 > P > 0.01$	almost significant*
$0.01 > P > 0.001$	significant**
$0.001 > P$	highly significant***

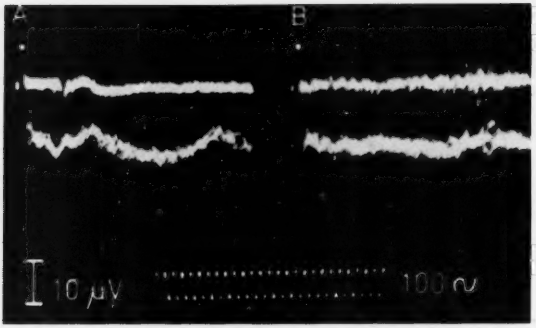

Fig. 2. The startle blink (upper tracings) and the non-specific EEG response (lower tracings) to a moderate auditory stimulus: *A*, during relaxation; *B*, during conversation with the investigator. In this and the following figures the white dot indicates the stimulus artefact.

Results

The Startle Blink Response and the Non-specific EEG Response

Preliminary experiments. Fig. 2 shows the startle blink reaction and the non-specific EEG response from the vertex to a moderate click stimulus during relaxation (A) and during lively conversation with the investigator (B). In *B* both responses have disappeared.

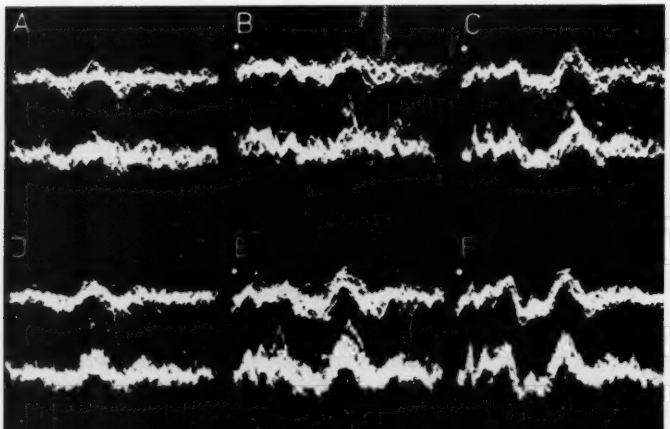

Fig. 3. EEG responses to left median nerve stimulation recorded from the vertex (upper tracings) and the scalp region corresponding to the contralateral somatosensory area (lower tracings): *A*, *B*, *C*, under relaxation; *D*, *E*, *F*, under reaction-speed condition. Stimulus strength: weak in *A* and *D*, moderate in *B* and *E*, strong in *C* a. *F*. In *A* and *D* the stimulus was given shortly before the start of the sweep.

Table III. The magnitude of the startle blink reaction to stimuli of different psychological significance

Mean values ± standard errors

Subject	Experimental Conditions			Individual average
	Reaction-speed High significance	Relaxation Moderate significance	Auto-stimulation Low significance	
S-ng	6.8 ± 0.8	4.6 ± 0.8	0.9 ± 0.7	4.1
K-n	16.8 ± 1.9	8.7 ± 2.0	1.2 ± 1.9	8.9
S-n	6.3 ± 0.8	1.5 ± 0.8	0.0 ± 0.8	2.6
F-i	6.0 ± 0.5	1.8 ± 0.4	0.3 ± 0.4	2.7
L-t	—	—	—	—
J-n	6.9 ± 0.9	0.6 ± 0.9	0.0 ± 0.9	2.5
Average	8.6 ± 1.0	3.4 ± 1.0	0.5 ± 1.0	4.2

Analysis of variance of mean values

Source of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio	Statistical significance
Between individuals	4	89.38	22.35	4.39	*
Between experimental situations	2	167.10	83.55	16.41	**
Residual	8	40.76	5.09	—	—
Total	14	297.24	—	—	—

The diminution of the responses when the subject's attention is distracted from the stimulus is in sharp contrast to the increase observed when his attention is intentionally drawn to it. This is illustrated in Fig. 3. The subject was informed that the stimulus was the signal in a reaction speed determination and he was instructed to press a button immediately he perceived the stimulus which was an electric shock to the left median nerve. The upper tracings show the non-specific EEG response recorded from the vertex at three different stimulus intensities under relaxation (A, B, C) and under the reaction speed condition (D, E, F). It is clear that all the superimposed responses in *D* and *E* have a large amplitude while only an occasional response in *A* and *B* is large. There is no distinct difference between the responses in *C* and *F*. Here, however, the stimulus was strong and it is probable that the response was already maximal under relaxation.

Quantitative measurements. The results would seem to show that the magnitudes of the non-specific EEG response and the startle blink reaction are related to

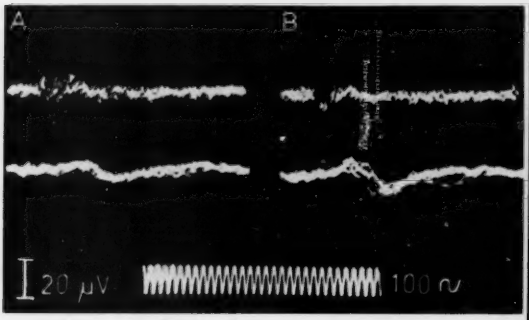

Fig. 4

Fig. 4. The non-specific EEG response (lower tracings) and the startle blink (upper tracings) evoked by *auditory* stimuli of moderate strength: *A*, under relaxation; *B*, when waiting to react to a *peripheral nerve* stimulus. The auditory stimulus started the sweep.

Fig. 5. The short-latency somatosensory response to peripheral nerve stimulation: *A*, under relaxation; *B*, under auto-stimulation.

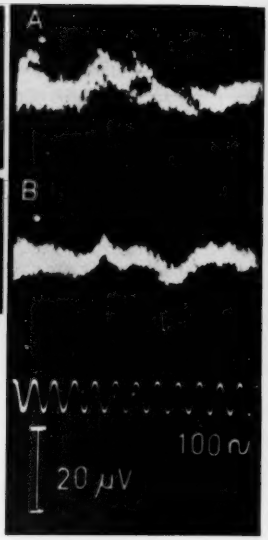

Fig. 5

the psychological significance of the stimulus. To obtain quantitative measurements of the results six of the subjects were investigated with auditory stimuli under each of the three experimental conditions. The responses were recorded and measured on single sweep tracings.

The mean magnitudes for each subject are shown in Table II and III. The responses were smaller under the auto-stimulation condition and larger under the reaction speed condition than under relaxation. The values obtained were treated by analysis of variance. The differences under the three experimental conditions are significant at the 0.1 per cent level for the EEG responses ($P < 0.001$) and at the 0.5 per cent level for the startle blink responses ($P < 0.005$).

Under the reaction speed condition it was observed that sometimes a subject reacted to stimuli other than the stimulus to which he was instructed to react. This seemed to occur especially at the beginning of the experiment. Experiments were therefore performed to determine whether the responses to these stimuli also were increased in magnitude. Fig. 4 A shows the response to a moderate auditory stimulus when the subject was relaxed. He was then given peripheral nerve stimuli to which he was instructed to react by pressing the button. In addition the auditory stimulus was given at irregular intervals. Fig. 4 B shows that the response to the auditory stimulus is now increased.

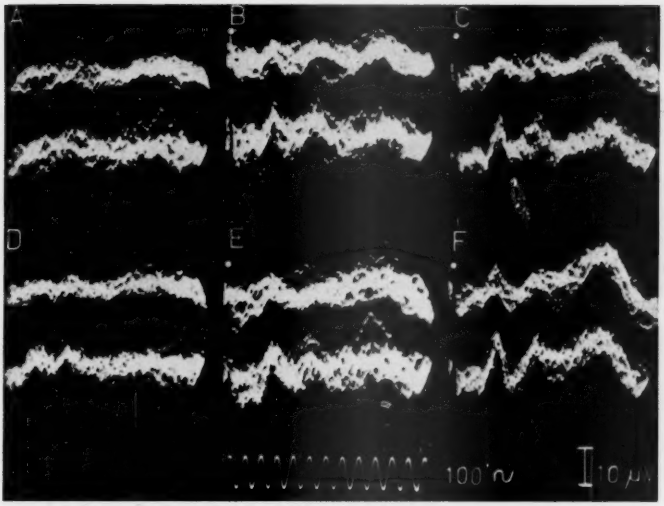

Fig. 6. Same as Fig. 3, but recorded on a second oscilloscope with a higher sweep speed.

The Short-Latency Somatosensory Response to Peripheral Nerve Stimulation

As is evident from the foregoing, the non-specific EEG response is very sensitive to the changes in experimental condition studied here. It is, of course, of interest to see whether the short-latency response to contralateral peripheral nerve stimulation described by DAWSON (1947) is also effected. This response, however, appears to be considerably more resistant.

Fig. 5 shows the short-latency somatosensory response under relaxation (A) and under auto-stimulation (B). In the latter the response appears to be somewhat decreased.

In Fig. 6, which is the same as Fig. 3 but displayed on a second oscilloscope with a faster sweep speed, the lower tracings show the short-latency response from the contralateral somatosensory area under relaxation (A, B, C) and under the reaction speed condition (D, E, F). The response in *D* shows a slight, possibly insignificant, increase as compared with that in *A*, while there is no difference between *B* and *E* and between *C* and *F*. This is in sharp contrast to the large increase in the long-latency responses recorded from both the vertex and the somatosensory area on the same occasion (Fig. 3). The relative insensitivity of the short-latency response is also demonstrated in Fig. 7 which shows the responses recorded from the vertex (upper tracings) and the somatosensory area contralateral to the side stimulated (lower tracings) under relaxation (A), auto-stimulation (B) and reaction speed (C). The first wave (between the arrows) in the somatosensory response is slightly diminished in

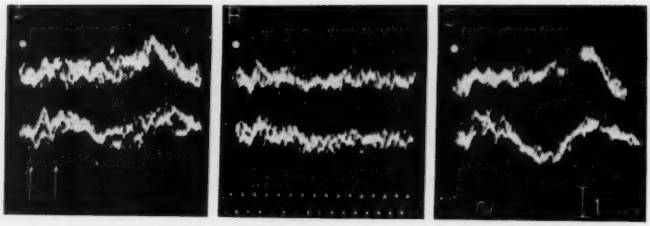

Fig. 7. EEG responses to left median nerve stimulation recorded from the vertex (upper tracings) and the scalp region corresponding to the contralateral somatosensory area (lower tracings) under *A*, relaxation, *B*, auto-stimulation and *C*, reaction-speed conditions. The arrows indicate the beginning and end of the short-latency somatosensory response.

Table IV. Duration of four respirations measured immediately before stimuli of different psychological significance

Mean values ± standard errors

Subject	Experimental Conditions			Individual average
	Reaction-speed High significance	Relaxation Moderate significance	Auto-stimulation Low significance	
S-ng	22.4 ± 0.1	24.8 ± 0.1	22.4 ± 0.1	23.2
K-n	20.5 ± 0.2	22.6 ± 0.2	19.1 ± 0.2	20.7
S-n	21.8 ± 0.4	20.7 ± 0.4	20.9 ± 0.4	21.1
F-i	22.1 ± 0.4	23.9 ± 0.3	19.5 ± 0.3	21.8
L-t	18.3 ± 0.2	20.4 ± 0.3	18.0 ± 0.3	18.9
J-n	22.1 ± 0.3	22.7 ± 0.3	20.9 ± 0.3	21.9
Average	21.2 ± 0.4	22.5 ± 0.4	20.1 ± 0.4	21.3
Rate	17.0	16.0	17.9	—

Analysis of variance of mean values

Source of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio	Statistical significance
Between individuals	5	31.09	6.22	7.32	**
Between experimental situations	2	17.10	8.55	10.06	**
Residual	10	8.52	0.85	—	—
Total	17	56.71	—	—	—

B but probably uninfluenced in *C*. This is in sharp contrast to the long-latency responses which are abolished in *B* and clearly increased in *C*.

Table V. Duration of 15 heart beats measured immediately before stimuli of different psychological significance

Mean values ± standard errors

Subject	Experimental Conditions			Individual average
	Reaction-speed High significance	Relaxation Moderate significance	Auto-stimulation Low significance	
S-ng	22.8 ± 0.7	23.0 ± 0.8	22.2 ± 0.8	22.7
K-n	17.6 ± 0.1	19.2 ± 0.1	16.4 ± 0.1	17.7
S-n	16.2 ± 0.1	16.4 ± 0.1	15.7 ± 0.1	16.1
F-i	21.6 ± 0.3	21.9 ± 0.3	18.1 ± 0.3	20.5
L-t	19.5 ± 0.2	20.9 ± 0.2	20.2 ± 0.2	20.2
J-n	15.6 ± 0.3	15.1 ± 0.3	15.5 ± 0.3	15.4
Average	18.9 ± 0.4	19.4 ± 0.4	18.0 ± 0.4	18.8
Rate	71.4	69.6	75.0	—

Analysis of variance of mean values

Source of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio	Statistical significance
Between individuals	5	119.70	23.94	27.84	***
Between experimental situations	2	5.99	3.00	3.49	0
Residual	10	8.61	0.86	—	—
Total	17	134.30	—	—	—

Cardiac and Respiratory Rates and the Spontaneous EEG Activity under the Three Experimental Conditions

Since it was evident that the three experimental conditions strongly influenced the non-specific EEG response and the startle blink reaction, it was considered of interest to see whether they also influenced other physiological functions. Measurements were therefore done of the heart and respiratory rates as well as of the EEG recorded from the posterior midline regions on the six subjects in Table II and III. The measurements were done over the period immediately preceding each stimulus and thus 20 to 22 measurements were done under each of the three experimental conditions on each subject. It should be stressed that the subjects remained in the same position throughout the entire investigation with their eyes open. Both the cardiac and the respiratory rates were slightly higher under the reaction speed condition than under relaxation and still somewhat higher under auto-stimulation (Table IV and

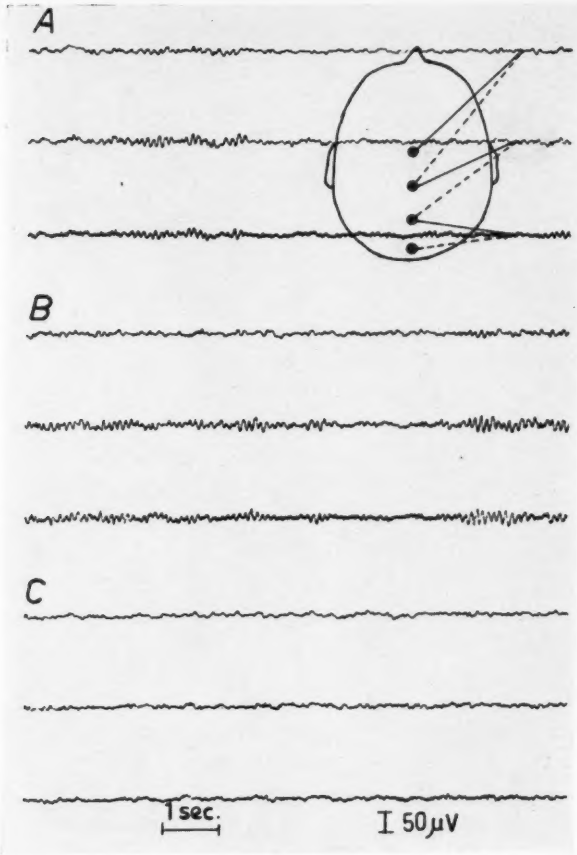

Fig. 8. Subject S-ng. EEG recorded from the posterior midline regions under *A*, relaxation, *B*, reaction-speed and *C*, auto-stimulation conditions. The eyes were open throughout.

V). The values obtained were treated by analysis of variance. The differences under the three experimental conditions are significant at the 1 per cent level for the respiratory rate ($P < 0.01$) and at the 5 per cent level for the cardiac rate ($P \approx 0.05$).

Fig. 8 shows the EEG recorded from the posterior midline region in the subject S-ng under each of the three experimental conditions. It is seen that there is a distinct difference in the amount of alpha waves under the two conditions in which it may be assumed that he was maximally alert, *i. e.* reaction speed and auto-stimulation, being greater in the former. The amount

Table VI. Number of alpha waves occurring over a 20-second period immediately before stimuli of different psychological significance

Mean values ± standard errors

Subject	Experimental Conditions			Individual average
	Reaction-speed High significance	Relaxation Moderate significance	Auto-stimulation Low significance	
S-ng.....	16.2 ± 1.5	7.6 ± 1.6	0.6 ± 1.5	8.1
K-n	5.7 ± 1.0	8.1 ± 1.0	0.9 ± 1.0	4.9
S-n	8.3 ± 0.9	8.1 ± 0.9	7.1 ± 0.9	7.8
F-i	51.0 ± 3.0	47.6 ± 3.0	7.9 ± 3.0	35.5
L-t	24.2 ± 2.2	17.1 ± 2.2	0.5 ± 2.2	13.9
J-n	24.0 ± 2.6	15.3 ± 2.6	3.5 ± 2.6	14.3
Average	21.6 ± 3.5	17.3 ± 3.5	3.4 ± 3.5	14.1

Analysis of variance of mean values

Source of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio	Statistical significance
Between individuals.....	5	1852.58	370.52	5.11	*
Between experimental situations	2	1080.75	540.38	7.45	*
Residual	10	725.10	72.51	—	—
Total	17	3658.43	—	—	—

of alpha waves under the reaction speed condition is even greater than under relaxation (Table VI). Two other subjects (L-t and J-n) showed essentially the same differences. Subjects K-n and F-i also had a larger amount of alpha waves under the reaction speed condition than under auto-stimulation. There was, however, no significant difference between the amount of alpha waves under the reaction speed condition and under relaxation. In the subject K-n there was no difference in the amount of alpha waves under the three experimental conditions. It should be pointed out, however, that he had very little activity in the alpha range. Analysis of variance was done on the values shown in Table VI. The differences in the amount of alpha activity under the three experimental conditions are significant at the 2.5 per cent level ($P < 0.025$).

There was no apparent difference in the frequency of the alpha waves under the three experimental conditions.

Discussion

The results show that the magnitudes of the startle blink and the non-specific EEG response are highly dependent on the changes in the experimental conditions described. If the assumptions made are correct, the responses are also correlated to changes in the psychological significance of the stimuli. It is possible that concepts such as different degrees of expectation or surprise would have been better than different degrees of psychological significance. It is evident, however, that these different terms have an overlapping meaning. For the present the essential thing is that changes in the psychological state which can be described by these terms are associated with changes in the magnitude of the responses studied. Other observations also support this deduction. The rapid adaptation of the startle blink (STRAUSS 1928, LANDIS and HUNT 1936) and the EEG response (BANCAUD, BLOCH and PAILLARD 1953, GASTAUT 1953) to repeated stimuli may be correlated to a reduction in the psychological significance of the stimulus. Likewise, the increase in amplitude when the evoking stimulus is combined with a strong stimulus, as in classical conditioning procedures (GASTAUT 1953, LARSSON 1956), may be correlated to an increase in the significance of the stimulus.

The results can be compared with the recent observations of HAGBARTH and KUGELBERG (1958) on the abdominal skin reflex which they demonstrated is a spinal reflex (KUGELBERG and HAGBARTH 1958). They found that psychological states such as attention, expectation and apprehension are associated with variations in the magnitude of the reflex. For example, whereas auto-stimulation evoked small reflexes, the reflexes evoked after a painful blow to the epigastrium were markedly increased. They consider that these effects are dependent on cerebral control of the excitability of the spinal interneurones which mediate the reflex.

The responses studied here are also of a relatively uncomplicated nature. The short-latency somatosensory response probably corresponds to the specific primary response recorded from the exposed cortex in animals. The non-specific EEG response is probably evoked by impulses mediated to the cortex via non-specific pathways in the brain stem and the startle blink probably has a reflex centre in the lower parts of the brain stem reticulum (see LARSSON 1956 for the literature). Evidence has been presented that centrifugal impulses from the cortex and brain stem reticulum exert a tonic influence on the specific afferent pathways (GRANIT 1954, LINDBLOM and OTTOSSON 1953, 1956, 1957, HAGBARTH and KERR 1954, HERNÁNDEZ-PEÓN and HAGBARTH 1955, HERNÁNDEZ-PEÓN *et al.* 1956 b, 1957 b, c, DAWSON 1958, APPELBERG *et al.* 1959). Since, however, the changes in experimental conditions had only a slight influence on the short-latency response recorded from the somatosensory region it is improbable that the changes in the startle blink and the non-specific EEG-response are secondary to a centrifugal influence on the specific

pathways. The centrifugal influence may, however, have more direct access to pathways in the brain stem reticulum (ADEY, SEGUNDO and LIVINGSTON 1957) to which these responses would seem to be intimately related.

The short-latency response recorded from the somatosensory area showed relatively little variation under the three experimental conditions set up in the present investigation. It is possible that more extreme experimental conditions would have produced greater differences. The difference in sensitivity between the short-latency response and the long-latency response is probably mostly of a quantitative nature. The relative insensitivity of the short-latency response as compared with the long-latency responses has been encountered during high frequency stimulation as well as during habituation procedures (LARSSON 1953, and unpublished observations). This is in agreement with the observation of HERNÁNDEZ-PEÓN *et al.* (1957 b) in the cat that mild distraction of attention abolished the secondary repetitive waves in the cortical evoked potentials at a time when the primary response was only slightly affected and, further (1957 c), that a large number of stimuli is necessary to habituate the responses in the specific afferent pathways. A similar observation was made by SHARPLESS and JASPER (1956). These workers found that the primary cortical response to auditory stimulation was increased rather than decreased at a time when the electroencephalographic blocking reaction was habituated. Whatever may be the cause of the difference in sensitivity, it would seem that part of the information that reaches the cortex is less influenced by changes in experimental conditions than the remaining part. Perhaps this is an advantage since it renders possible the dividing of the inflowing information into what one may call relatively objective information and into subjectively coloured information suitable for the current need.

If one considers the experimental conditions in the order of auto-stimulation, reaction-speed and relaxation, it is seen that the cardiac and respiratory rates show a distinct tendency to decrease. The EEG record is "desynchronized" under auto-stimulation, contains a large amount of alpha waves under the reaction-speed condition and a lesser amount under relaxation. According to current terminology, the EEG findings can be said to express a declining level of "cortical excitatory state". The concept which lies behind this expression must be regarded as poorly defined. Here, however, it shall merely be noted that a declining level of "cortical excitatory state" expressed with the aid of the EEG appears to be correlated to a falling cardiac and respiratory rate.

The EEG tended to show a large amount of alpha waves under the reaction-speed condition in contrast to the "desynchronization" under auto-stimulation. According to current conceptions this would mean that the subjects were more awake under auto-stimulation. It is, however, impossible to differentiate between the levels of wakefulness under the two experimental conditions. When is the subject more awake, when he is carrying out a fairly

simple task or when he is prepared to react to a stimulus? There is no doubt that he is wide awake in the latter event which is shown, for one thing, in the tendency to react to stimuli to which he was not instructed to react. The two experimental conditions would seem to produce a difference in the span of attention rather than in the degree of attention. Accordingly, the common view that a high degree of wakefulness is invariably accompanied by desynchronization of the EEG record is not wholly correct.

Synchronization of the EEG record in non-relaxed states has, however, been reported. GREEN and ARDUINI (1954) observed in various experimental animals that arousal was accompanied by synchronization of the hippocampal record. This was invariably associated with "desynchronization" of the cortical record and consequently cannot be considered directly comparable to the results obtained here. LOOMIS, HARVEY and HOBARTH (1937), however, found that an intense listening for a barely audible sound may increase the alpha rhythm and WILLIAMS (1939) reported that psychological states such as awareness, attentiveness and readiness may be associated with a large amount of alpha waves.

An observation made by YOSHII and TSUKIYAMA (1952) would seem to be relevant in this connection. These workers studied the EEG in rats running in a T-maze in order to find a reward in one of the arms of the T. Just before or immediately after the start of the run there was an augmentation of the basic waves which persisted until the rat reached the point at which it had to decide the direction to continue. When the rat approached the reward the activity became "desynchronized". There would seem to be a similarity between the psychological state of the rat before it selects its route and the expectant state of a human being ready to react to a stimulus.

Experiments described by SIMON and EMMONS (1956) are also of considerable interest. These workers recorded the alpha rhythm from the occiput and vertex during verbal stimuli in waking and sleeping subjects. They found that the amount of alpha waves at the time of stimulation was directly correlated to the subject's ability to respond to the stimuli and correctly recall them later.

Accordingly, observations reported in the literature as well as those made in the present investigation indicate that psychological states in which the subject is well prepared to react to peripheral stimuli may be associated with a large amount of alpha waves.

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